STUDIES ON ISOZYMES LOCALIZED IN BOTH PEROXISOMES AND MITOCHONDRIA OF *N*-ALKANE-UTILIZABLE YEAST, *CANDIDA TROPICALIS*

HIROYUKI KAWACHI

1997

STUDIES ON
ISOZYMES LOCALIZED IN
BOTH PEROXISOMES AND MITOCHONDRIA
OF *N*-ALKANE-UTILIZABLE YEAST,
CANDIDA TROPICALIS

HIROYUKI KAWACHI

1997

PREFACE

This is a thesis submitted by the author to Kyoto University for the degree of Doctor of Engineering. The studies collected here have been carried out under the direction of Professor Atsuo Tanaka in the Laboratory of Industrial Biochemistry, Department of Industrial Chemistry and in the Laboratory of Applied Biological Chemistry, Department of Synthetic Chemistry and Biological Chemistry, and Graduate School of Engineering, Kyoto University, during 1990-1997.

The author wishes to express grateful acknowledgement to Professor Atsuo Tanaka for his kind guidance and warm encouragement throughout this work.

The author also greatly appreciates Associate Professor Mitsuyoshi Ueda for his useful discussion and continuous helpful support during the course of this work.

Sincere gratitude is also extended to Assistant Professor Haruyuki Atomi for his invaluable advice and continuous encouragement throughout this work.

The author is deeply grateful to Ms. Setsuko Yamamoto, Mr. Shin-ichi Sanuki, Mr. Toshiya Imajo, and Ms. Kaori Shimizu for their helpful discussions and kind assistance.

The author's thanks are also due to Professor Masako Osumi, Ms.

Naomi Kamasawa, Ms. Tomoko Yoshida, Ms. Keiko Kobayashi, and Ms. Noriko Hashimoto of Japan Women's University for their experimental work and valuable discussion.

Finally, the author wishes to express deeply thanks to all members of Professor Tanaka's Laboratory for their kind advice and support on this work.

Hiroyuki Kawachi

Division of Molecular Neurobiology,

National Institute for Basic Biology

Abbreviations

ADH1	*Saccharomyces cerevisiae* alcohol dehydrogenase
CAT	carnitine acetyltransferase
CCA1	*Saccharomyces cerevisiae* ATP(CTP):tRNA nucleotidyltransferase
CIT	citrate synthase
FOX3	*Saccharomyces cerevisiae* 3-ketoacyl CoA thiolase
FUM1	*Saccharomyces cerevisiae* fumarase
HTS1	*Saccharomyces cerevisiae* histidyl-tRNA synthetase
IDH	isocitrate dehydrogenase
LEU4	*Saccharomyces cerevisiae* α-isopropylmalate synthase
MOD5	*Saccharomyces cerevisiae* Δ^2-isopentenyl pyrophosphate:tRNA isopentenyltransferase
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
PCR	polymerase chain reaction
PEX	peroxin
POX4	*Candida tropicalis* acyl-CoA oxidase
PTS	peroxisomal targeting signal
RACE	rapid amplification of cDNA ends
RPA	RNase protection assay
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
SPT	serine:pyruvate aminotransferase
SUC2	*Saccharomyces cerevisiae* invertase
TCA cycle	tricarboxylic acid cycle
TRM1	*Saccharomyces cerevisiae* N^2,N^2-dimethylguanosine-specific tRNA methyltransferase
UPR-ICL	*Candida tropicalis* isocitrate lyase promoter
VAS1	*Saccharomyces cerevisiae* valyl-tRNA synthetase

CONTENTS

INTRODUCTION

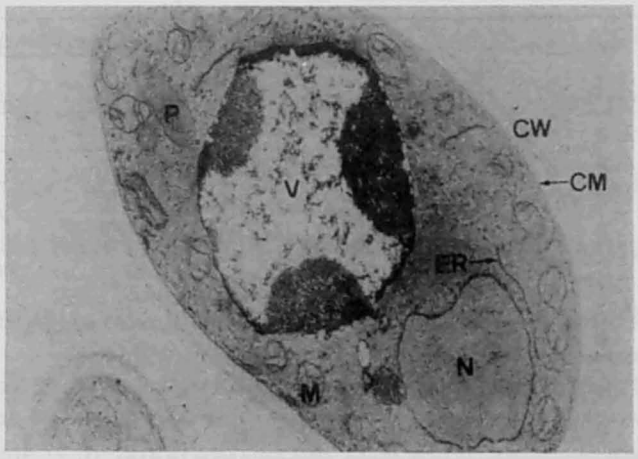
One of the most important features that distinguishes eukaryotic from prokaryotic cells is their segregation of specific metabolic reactions within separate intracellular compartments. The nature of each compartment is determined by the proteins that it contains. Most of proteins are encoded by nuclear genes, and are transported into their compartment by the targeting sequences that are specific to each compartment, such as nuclei, endoplasmic reticula, mitochondria, and peroxisomes (1-4). Specific proteins are usually found at only one location. However, the segregation of specific metabolic reactions within specific intracellular locations is not absolute and the presence of isozymes that function in more than one cellular compartment have been known. It is of great interest to clarify the sorting mechanisms of such isozymes.

The author's studies deal with isozymes localized in both peroxisomes and mitochondria of *n*-alkane-utilizable yeast *Candida tropicalis,* to obtain some basal biochemical information on the targeting mechanisms of isozymes to both subcellular organelles.

Assimilation pathway of *n*-alkane in the yeast, *Candida tropicalis*

In *Candida tropicalis* cells grown on *n*-alkane, whose carbon chain-length is between 10 and 13, a large numbers of peroxisomes were observed (Fig. 1) (5, 6). This fact suggests that peroxisomes might play important roles in alkane assimilation. A possible metabolic function of peroxisomes in alkane-utilizing yeast is summarized in Fig. 2. *n*-Alkane incorporated by the cells is first oxidized to alcohols in microsomes. This reaction is supposed to be catalyzed by cytochrome P-450 and cytochrome P-450 reductase (7). Alcohols are transported into peroxisomes, and are oxidized *via* aldehydes to fatty acids by NAD-linked alcohol dehydrogenase and aldehyde dehydrogenase (8). Fatty acids are activated to CoA esters by acyl-CoA synthetase (8) and then degraded by the fatty acid β-oxidation system to yield acetyl-CoA (9,10). Enzymes that participate in the fatty acid β-oxidation system are acyl-CoA oxidase (11) coupled to catalase (12), a bifunctional enzyme (enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase) (13), 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase (14). Acetyl-CoA is further metabolized through the tricarboxylic acid cycle (TCA cycle) or the glyoxylate cycle. In the TCA cycle, acetyl-CoA is condensed with oxalacetate by citrate synthase to yield citrate and citrate is metabolized to isocitrate. Isocitrate is degraded to 2-oxoglutarate by isocitrate dehydrogenase. After citrate is metabolized through the TCA cycle to produce oxalacetate, releasing two molecules of CO_2 and providing reducing powers which are used to generate ATP by

A



B

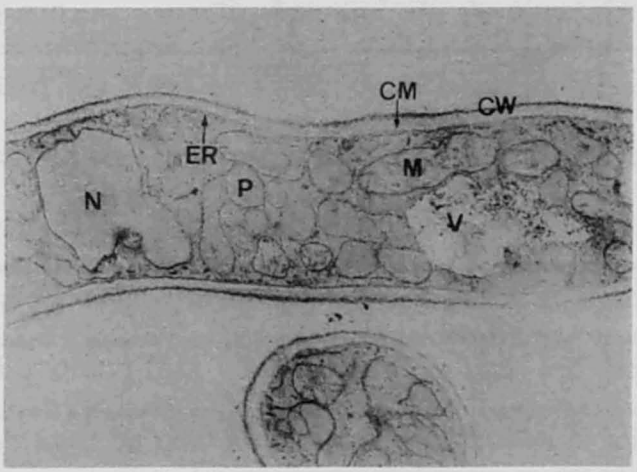


Fig. 1. Electronmicrographs of glucose-grown (A) and *n*-alkane-grown cells (B) of *Candida tropicalis* pK233.

CM, cell membrane; CW, cell wall; ER, endoplasmic reticulum; M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole

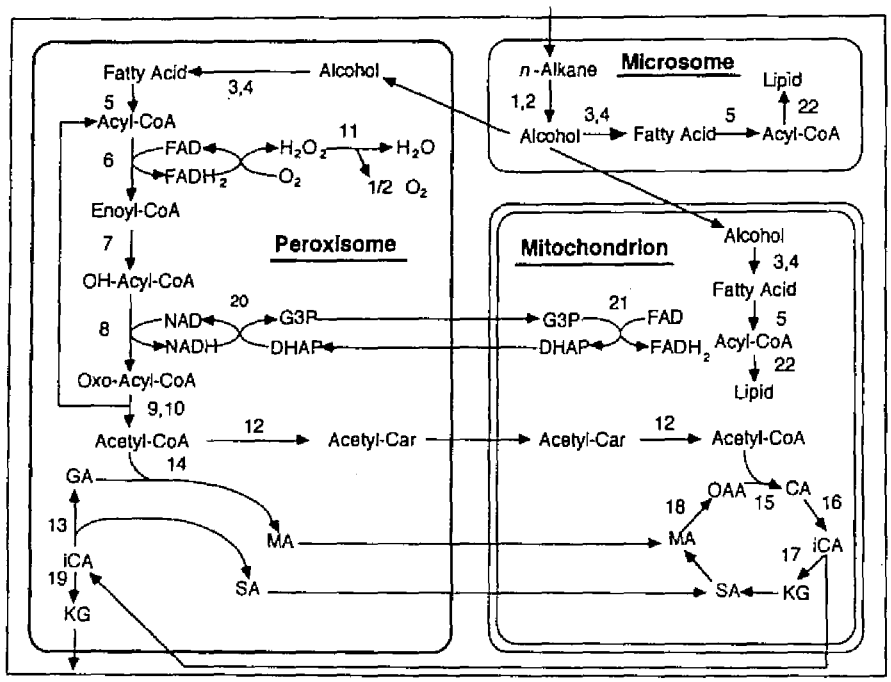


Fig. 2. Possible roles of peroxisomes in connection with those of mitochondria and microsomes in alkane assimilation by yeasts.

Enzymes: 1, cytochrome P-450; 2, NADPH-cytochrome P-450 (cytochrome c) reductase; 3, alcohol dehydrogenase; 4, aldehyde dehydrogenase; 5, acyl-CoA synthetase; 6, acyl-CoA oxidase; 7, enoyl-CoA hydratase; 8, 3-hydroxyacyl-CoA dehydrogenase; 9, 3-ketoacyl-CoA thiolase; 10, acetoacetyl-CoA thiolase; 11, catalase; 12, carnitine acetyltransferase; 13, isocitrate lyase; 14, malate synthase; 15, citrate synthase; 16, aconitase; 17, NAD-dependent isocitrate dehydrogenase; 18, malate dehydrogenase; 19, NADP-dependent isocitrate dehydrogenase; 20, NAD-dependent glycerol-3-phosphate dehydrogenase; 21, FAD-dependent glycerol-3-phosphate dehydrogenase; 22, glycerophosphate acyltransferase

Abbreviations: Acetyl-Car, acetylcarnitine; CA, citrate; DHAP, dihydroxyacetone phosphate; GA, glyoxylate; G3P, glycerol-3-phosphate; iCA, isocitrate; KG, 2-oxoglutarate; MA, malate; OAA, oxalacetate; SA, succinate

oxidative phosphorylation connected to the respiratory chain. On the other hand, the glyoxylate cycle plays an important role in the biosynthesis of cellular components, including carbohydrates and amino acids. This cycle shares most of its enzymes with the TCA cycle, except for two distinct enzymes, isocitrate lyase and malate synthase. Isocitrate lyase catalyzes the cleavage of isocitrate into succinate and glyoxylate. Glyoxylate is then condensed with acetyl-CoA by malate synthase to produce malate. Either of succinate or malate is metabolized *via* the TCA cycle again. In this cycle, the net synthesis of C_4-compounds is accomplished from two molecules of acetyl-CoA. In *n*-alkane-utilizing yeast *Candida tropicalis*, the fatty acid β-oxidation system and key enzymes of the glyoxylate cycle (isocitrate lyase and malate synthase) are localized only in peroxisomes. Other glyoxylate cycle enzymes which are common to the TCA cycle, such as citrate synthase and malate dehydrogenase, are localized in mitochondria, but not in peroxisomes. In order to metabolize acetyl-CoA through the TCA cycle and the glyoxylate cycle, acetyl-CoA must be transported from peroxisomes to mitochondria. Therefore, peroxisomal and mitochondrial carnitine acetyltransferases constitute an acetylcarnitine shuttle system between these organelles (15). In this way peroxisomes and mitochondria cooperate with each other in order to metabolize alkanes efficiently. Besides these enzymes, NADP-linked isocitrate dehydrogenase (NADP-IDH) localizes in peroxisomes (16). There have been no reports of an NADP-IDH localized in peroxisomes in other organisms as

of yet, and its precise metabolic functions are not well understood. Isocitrate lyase and isocitrate dehydrogenase compete the same substrate, isocitrate. Control mechanisms to share the substrate may operate between both enzymes.

Mitochondria and protein transport into mitochondria

Mitochondria are the essential compartment of all eukaryotic cells, making ATP by utilizing energy obtained from the oxidation of substances. The mitochondrial subcompartments, consisting of outermembrance, intermembrance space, inner membrance, and matrix, each contain a specific set of in total about 700 different proteins. Although mitochondria have a genome of their own, most mitochondrial proteins are encoded in nuclei. The precursors of nuclear DNA encoded mitochondrial proteins are synthesized on cytosolic ribosomes and are posttranslationally imported into mitochondria (3, 17, 18). Precursors cotain targeting sequences of 10 to 70 amino acid residues, which in most cases are localized at the N-termini as cleavable presequences (19, 20). Mitochondrial targeting signals are rich in positively charged residues and essentially lack acidic amino acids (21, 22). They are necessary and sufficient to direct nonmitochondrial passenger proteins into mitochondrial matrix (23, 24). In general, the targeting signals share no sequence homology, but many of them have the potential to form amphiphilic α helices or β seets (that is, secondary structural arrangements with the

positively charged and the hydrophobic residues being exposed to opposite faces) (25). This amphiphilicity may be important for the initial membrance insertion or the interaction with specific receptor proteins at the mitochondrial surface, or both. The precursor proteins bind to the mitochondrial surface and are transported across outer and inner membrance at sites where the two membrances are closely apposed (26). Passage across the two membrances requires for the proteins to be loosely folded. Once the proteins have reached matrix, their targeting sequences are removed and the mature polypeptides fold into their native conformation (17, 26). Within recent years, more than 20 different proteins have been identified to be involved in this mitochondrial protein import machinery (27). In the present study, the author has isolated, and sequenced mitochondrial proteins in order to compare the details on the mitochondrial and peroxisomal protein import machineries in *Candida tropicalis*.

Peroxisomes and protein transport into peroxisomes

Peroxisomes were first discovered by electron microscopy in rat kidney in 1954 by Rhodin (28). These subcellular organelles are morphologically characterized by a single lipid bilayer membrance, a fine granular matrix. As for the physiological role, Lazarow and de Duve found the fatty acid β-oxidation system in rat liver peroxisomes (29), which had been believed to be present

only in mitochondria. Peroxisomal matrix proteins are synthesized on free ribosomes in cytosol and are posttranslationally imported into the organelle (30), just like mitochondrial proteins. At least three peroxisomal targeting signals (PTSs) are implicated in the transport of proteins into peroxisomal matrix. The major targeting signal, PTS1, used by a lot of matrix proteins, is comprised of the C-terminal tripeptides, -Ser-Lys-Leu, or variations of this sequence (31, 32). The second peroxisomal targeting sequence, PTS2, has been found on the N-termini of 3-ketoacyl-CoA thiolase (33, 34) and other proteins (35-37). These sequences are 16-26 amino acids in length and may or may not be cleaved upon import. Still many other peroxisomal proteins, for example, *Candida tropicalis* acyl-CoA oxidase (38) and *Saccharomyces cerevisiae* catalase A (39), contain redundant internal targeting sequences.

Recently, the molecular identification and characterization of the machineries that are responsible for the peroxisomal protein import represent a major aim. Mutants in the import of peroxisomal proteins have been isolated from several species (40, 41). Analysis of these mutants has led to the identification of factors required for import. These factors include receptors, peroxin 5 (PEX5) for PTS1 proteins (42-45), and PEX7 for PTS2 proteins (42, 46, 47).

Candida tropicalis is one of ideal tools for the research of peroxisomes because the development of peroxisomes can be easily controlled by changing

the carbon source and a large number of peroxisomes appear in the cells grown on *n*-alkanes. Analysis of the gene structure for the peroxisomal proteins would contribute to understand the mechanisms of protein transport into peroxisomes.

Sorting of isozymes to more than one intracellular compartment

In several instances, specific enzymes have been located in more than one subcellular compartment. Several mechanisms have evolved to target proteins to different intracellular compartments. The most common mechanism is that each protein is encoded by a different gene. For instance, citrate synthases in *Saccharomyces cerevisiae* are encoded by two genes, *ScCIT1* and *ScCIT2* (48, 49). *ScCIT1* has been identified as the gene encoding the mitochondrial citrate synthase and *ScCIT2* as the gene encoding the peroxisomal enzyme. However, an increasing number of multi-compartmentalized proteins that are encoded by a single gene are identified. These mechanisms include alternative forms of transcription initiation and translation initiation. First, some genes can produce more than one mRNA by the use of alternative transcription initiation sites (Fig. 3A). Different transcripts encoded by the same gene have the potential to encode different targeting information, with the result that the polypeptides encoded by these transcripts can be localized to different intracellular compartments. In the

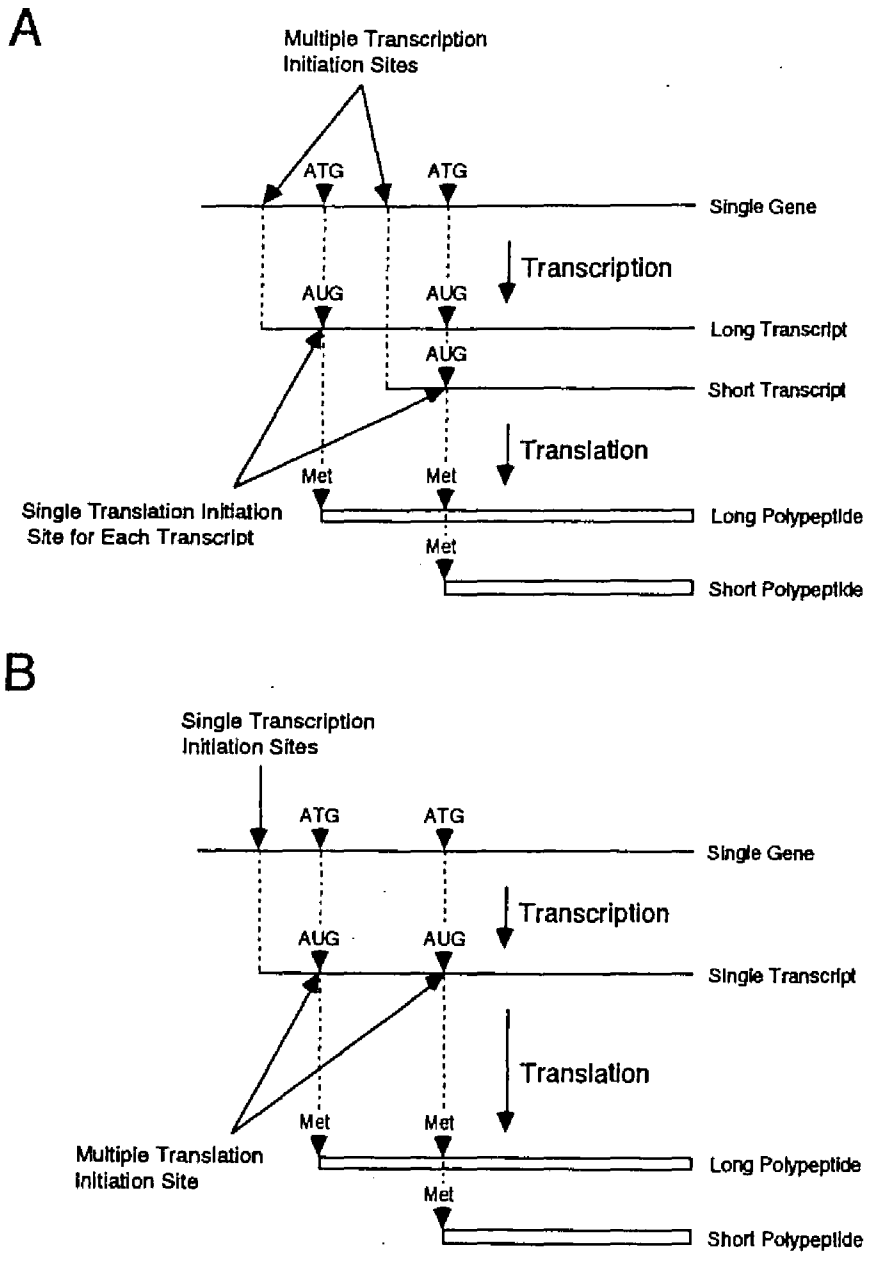


Fig. 3. Schematic representation of alternative transcription initiation (A) and alternative translation initiation (B).

case of *SUC2* encoding invertase, the longer mRNA codes for the secreted form of the enzyme, whereas the shorter mRNA encodes the cytoplasmic form of the enzyme (50, 51). In the case of *HTS1* (52), *FUM1* (53), *VAS1* (54), and *LEU4* (55), on the other hand, the longer mRNAs encode the mitochondrial enzymes and the shorter mRNAs the cytoplasmic enzymes. Such dual targeting is also found in rat serine: pyruvate aminotransferase (56) and in *Saccharomyces cerevisiae* carnitine acetyltransferase (57), which are targeted to both mitochondria and peroxisomes. Another mechanism by which variability can be introduced into the N-terminus of a polypeptide encoded by a single gene is alternative translation initiation from a single transcript (Fig. 3B). Alternative translation initiation has been suggested to occur in the case of the mitochondrial and cytosolic localization of rat liver fumarase (58), the mitochondrial, nuclear, and cytosolic localization of *Saccharomyces cerevisiae MOD5* (59) and *CCA1* (60), and the mitochondrial and peroxisomal localization of feline alanine: glyoxylate aminotransferase (61). The mechanisms involved in alternative translation initiation are not understood. Leaky ribosome scanning might be resulted from a poor Kozak configuration (62) arround the first site, compared with the second, or the secondary structure of the mRNA might dictate that the second site is much more accessible than the first.

In the present work, the author has isolated and sequenced the gene

for peroxisomal NADP-linked isocitrate dehydrogenase for the first time. The author has compared its structure with previously reported NADP-linked isocitrate dehydrogenases from various sources. Moreover, the author has also investigated further NADP-linked isocitrate dehydrogenase isozymes in *Candida tropicalis*, including mitochondrial type and cytosolic type, which has been found in other organisms. In the case of mitochondrial and peroxisomal carnitine acetyltransferases, on the other hand, these isozymes have been already purified and their kinetic properties have been investigated (63). The author has examined whether they are encoded by one gene or two distinct genes, and discussed the sorting mechanism of these isozymes. Eukaryotic cells have evoluted a number of mechanisms to target proteins with the same or similar functions to different intracellular locations. Thus, investigation on the isozymes is very interesting from the viewpoint of molecular evolution.

REFERENCES

1. Dingwall, C. & Laskey, R.A. (1986) *Annu. Rev. Cell Biol.* **2**, 367.
2. Pfeffer, S.R. & Rothman, J.E. (1987) *Annu. Rev. Biochem.* **56**, 829.
3. Hartl, F.-U., Pfanner, N., Nicholson, D.W. & Neupert, W. (1989) *Biochim. Biophys. Acta* **998**, 1.
4. Gould, S.J., Keller, G.-A. & Subramani, S. (1988) *J. Cell Biol.* **107**, 897.

5. Osumi, M., Miwa, N., Teranishi, Y., Tanaka, A. & Fukui, S. (1974) *Arch. Microbiol.* **99**, 181.

6. Osumi, M., Fukuzumi, F., Teranishi, Y., Tanaka, A. & Fukui, S. (1975) *Arch. Microbiol.* **103**, 1.

7. Duppel, W., Lebeault, J.M. & Coon, M.J. (1973) *Eur. J. Biochem.* **36**, 583.

8. Yamada, T., Nawa, H., Kawamoto, S., Tanaka, A. & Fukui, S. (1980) *Arch. Microbiol.* **128**, 145.

9. Kawamoto, S., Nozaki, C., Tanaka, A. & Fukui, S. (1978) *Eur. J. Biochem.* **83**, 609.

10. Ueda, M., Yamanoi, K., Morikawa, T., Okada, H. & Tanaka, A. (1985) *Agric. Biol. Chem.* **49**, 1821.

11. Osumi, T. & Hashimoto, T. (1978) *Biochem. Biophys. Res. Commun.* **83**, 479.

12. Yamada, T., Tanaka, A. & Fukui, S. (1982) *Eur. J. Biochem.* **125**, 517.

13. Ueda, M., Morikawa, T., Okada, H. & Tanaka, A. (1987) *Agric. Biol.Chem.* **51**, 2197.

14. Kurihara, T., Ueda, M. & Tanaka, A. (1989) *J. Biochem.* **106**, 474.

15. Kawamoto, S., Ueda, M., Nozaki, C., Yamamura, M., Tanaka, A. & Fukui, S. (1978) *FEBS Lett.* **96**, 37.

16. Kawamoto, S., Tanaka, A., Yamamura, M., Teranishi, Y., Fukui, S. &

Osumi, M. (1977) *Arch. Microbiol.* **112**, 1.

17. Attardi, G. & Schatz, G. (1988) *Annu. Rev. Cell Biol.* **4**, 289.
18. Pfanner, N., Hartl, F.-U. & Neupert, W. (1988) *Eur. J. Biochem.* **175**, 205.
19. Harmey, M.A. & Neupert, W. (1979) *FEBS Lett.* **108**, 385.
20. Maccecchini, M.-L., Rudin, Y., Blobel, G. & Schatz, G. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 343.
21. Viebrock, A., Prez, A. & Sebald, W. (1982) *EMBO J.* **1**, 565.
22. Kaput, J., Goltz, S. & Blobel, G. (1982) *J. Biol. Chem.* **257**, 15054.
23. Hart, E.C., Pesold-Hurt, B. & Schatz, G. (1984) *FEBS Lett.* **178**, 306.
24. Horwich, A.L., Kalousek, F., Mellman, I. & Rosenberg, L.E. (1985) *EMBO J.* **4**, 1129.
25. Roise, D., Theiler, F., Horvath, S.J., Tomich, J.M., Richards, J.H., Allison, D.S. & Schatz, G. (1988) *EMBO J.* 7, 649.
26. Hartl, F.-U. & Neupert, W. (1990) *Science* **247**, 930.
27. Kubrich, M., Dietmeier, K. & Pfanner, N. (1995) *Curr. Genet.* **27**, 393.
28. de Duve, C. & Baudhuin, P. (1966) *Physiol. Rev.* **46**, 323.
29. Lazarow, P.B. & de Duve, C. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2043.
30. Lazarow, P.B. & Fujiki, Y. (1985) *Annu. Rev. Cell Biol.* **1**, 489.
31. Gould, S.J., Keller, G.-A., Hosken, N., Wilkinson, J. & Subramani, S.

(1989) *J. Cell Biol.* **108**, 1657.

32. Gould, S.J., Krisans, S., Keller, G.-A. & Subramani, S. (1990) *J. Cell Biol.* **110**, 27.
33. Swinkels, B.W., Gould, S.J., Bodnar, A.G., Rachubinski, R.A. & Subramani, S. (1991) *EMBO J.* **10**, 3255.
34. Erdmann, R. (1994) *Yeast* **10**, 935.
35. Gietl, C. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5773.
36. Faber, K.N., Keizer-Gunnink, I., Pluim, D., Harder, W., AB, G. & Veenhuis, M. (1995) *FEBS Lett.* **357**, 115.
37. Waterham, H.R., Titorenko, V.I., Haima, P., Cregg, J.M., Harder, W. & Veenhuis, M. (1994) *J. Cell Biol.* **127**, 737.
38. Kamiryo, T., Sakasegawa, Y. & Tan, H. (1989) *Agric. Biol. Chem.* **53**, 179.
39. Kragler, F., Langeder, A., Raupachova, J., Binder, M. & Hartig, A. (1993) *J. Cell Biol.* **120**, 665.
40. Kunau, W.-H., Beyer, A., Franken, T., Gotte, K., Marzioch, M., Saidowsky, J., Skaletz-Rorowski, A. & Wiebel, F.F. (1993) *Biochimie* **75**, 209.
41. Lazarow, P.B. (1993) *Trends Cell Biol.* **3**, 89.
42. Distel, B., Erdmann, R., Gould, S.J., Blobel, G., Crane, D.I., Cregg, J.M., Dodt, G., Fujiki, Y., Goodman, J.M., Just, W.W., Kiel, J.A.K.W., Kunau, W.-H., Lazarow, P.B., Mannaerts, G.P., Moser, H.W., Osumi,

T., Rachubinski, R.A., Roscher, A., Subramani, S., Tabak, H.F., Tsukamoto, T., Valle, D., van der Klei, I. & van Veldhoven P.P. (1996) *J. Cell Biol.* **135**, 1.

43. Van Der Leij, I., Franse, M.M., Elgersma, Y., Distel, B. & Tabak, H.F. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 11782.
44. McCollum, D., Monosov, E. & Subramani, S. (1993) *J. Cell Biol.* **121**, 761.
45. Fransen, M., Brees, C., Baumgart, E., Vanhooren, J. C. T., Baes, M., Mannaerts, G.P. & Van Veldhoven, P.P. (1995) *J. Biol. Chem.* **270**, 7731.
46. Morzioch, M., Erdmann, R., Veenhuis, M. & Kunau, W.-H. (1994) *EMBO J.* **13**, 4908.
47. Zhang, J. W. & Lazarow, P.B. (1995) *J. Cell Biol.* **129**, 65.
48. Suissa, M., Suda, K. & Schatz, G. (1984) *EMBO J.* **3**, 1773.
49. Rozenkrantz, M., Alam, T., Kim, K.-S., Clark, B.J., Stere, P.A. & Guarente, L.P. (1986) *Mol. Cell. Biol.* **6**, 4509.
50. Carson, M. & Botstein, D. (1982) *Cell* **28**, 145.
51. Perlman, D., Halvorson, H.O. & Cannon, L.E. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 781.
52. Natsoulis, G., Hilger, F. & Fink, G.R. (1986) *Cell* **46**, 235.
53. Wu, M. & Tzagoloff, A. (1987) *J. Biol. Chem.* **262**, 12275.

54. Chatton, B., Walter, P., Ebel, J.-P., Lacroute, F. & Fasiolo, F. (1988) *J. Biol. Chem.* **263**, 52.

55. Beltzer, J.P., Morris, S.R. & Kohlhaw, G.B. (1988) *J. Biol. Chem.* **263**, 368.

56. Oda, T., Funai, T. & Ichiyama, A. (1990) *J. Biol. Chem.* **265**, 7513.

57. Elgersma, Y., Van Roermund, C.W.T., Wanders, R.J.A. & Tabak, H.F. (1995) *EMBO J.* **14**, 3472.

58. Suzuki, T., Yoshida, T. & Tuboi, S. (1992) *Eur. J. Biochem.* **207**, 767.

59. Slusher, L.B., Gillman, E.C., Martin, N.C. & Hopper, A.K. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9789.

60. Wolfe, C.L., Lou, Y.-C., Hopper, A.K. & Martin, N.C. (1994) *J. Biol. Chem.* **269**, 13361.

61. Lumb, M.J., Purdue, P.E. & Danpure, C.J. (1994) *Eur. J. Biochem.* **221**, 53.

62. Kozak, M (1991) *J. Biol. Chem.* **266**, 19867.

63. Ueda, M., Tanaka, A. & Fukui, S. (1982) *Eur. J. Biochem.* **124**, 205.

SYNOPSIS

Part I Mitochondrial and peroxisomal NADP-linked isocitrate dehydrogenases in *Candida tropicalis* encoded by distinct genes

Isocitrate dehydrogenase (IDH) catalyzes the oxidative decarboxylation of isocitrate to 2-oxoglutalate. In eukaryotic cells, IDHs are classified as NAD-linked enzymes (NAD-IDH) present in mitochondria and NADP-linked enzymes (NADP-IDH) present in cytosol and/or mitochondria. *Candida tropicalis* posesses two IDHs. One is NAD-IDH localized in mitochondria and another is NADP-IDH localized in peroxisomes. There have been no reports of NADP-IDH localized in peroxisomes in other organisms as of yet.

In Chapter 1, the author showed the presence of the third type of IDH, mitochondrial NADP-IDH in *Candida tropicalis*. One gene encoding mitochondrial NADP-IDH included an open reading frame of 1,290 bp corresponding to 430 amino acids. When this gene was introduced into *Saccharomyces cerevisiae* NADP-IDH activity was highly induced. The molecular mass of the purified recombinant protein was distinct from that

observed for peroxisomal NADP-IDH purified from *n*-alkane-grown *Candida tropicalis* cells. Polyclonal antibodies raised against the recombinant mitochondrial NADP-IDH showed little cross-reaction against peroxisomal NADP-IDH, suggesting the presence of another gene responsible for peroxisomal NADP-IDH in *Candida tropicalis*. Similar levels of mitochondrial NADP-IDH mRNA and protein could be detected in cells regardless of the growth substrate.

Chapter 2 deals with the isolation of the gene encoding peroxisomal NADP-IDH. Using the cDNA fragment specific for peroxisomal NADP-IDH as a probe, one peroxisomal NADP-IDH gene was isolated. This gene consisted of 1,233 bp, corresponding to 411 amino acids. This enzyme had no typical C-terminal peroxisomal targeting signal. However, near the N-terminal region, a sequence with high similarity with both the putative N-terminal peroxisomal tergeting sequence of 3-ketoacyl CoA thiolase of *Saccharomyces cerevisiae* and the internal peroxisomal targeting sequence of acyl-CoA oxidase of *Candida tropicalis* was found. Northern blot analysis revealed that mRNA levels of peroxisomal NADP-IDH inceased in *n*-alkane-grown cells compared to glucose-grown cells, different from the case of mitochondrial NADP-IDH.

Part II *Candida tropicalis* mitochondrial and peroxisomal carnitine acetyltransferases synthesized from one gene by alternative

translation initiation

In yeast, degradation of fatty acids takes place exclusively in peroxisomes, in which they are oxidized completely to acetyl-CoA. Carnitine acetyltransferases (CAT) localized in peroxisomes and mitochondria form an "acetylcarnitine shuttle", allowing the transfer of acetyl unit from peroxisomes to mitochondria in order to complete the operation of TCA cycle in mitochondria. These two peroxisomal and mitochondrial CATs have already been purified from *Candida tropicalis* and their kinetic properties were determined.

To obtain more details on the structures of these isozymes, the author has isolated and sequenced the gene encoding CATs as described in Chapter 1. One gene was isolated from the *Candida tropicalis* genomic DNA library. Sequence analysis disclosed that the open reading frame was 1,881 bp, corresponding to 627 amino acids. When the gene was introduced under the control of its own promoter into *Saccharomyces cerevisiae*, CAT activity in the cells was enhanced. Western blot analysis revealed the presence of two proteins whose sizes corresponded to peroxisomal (68 kDa) and mitochondrial (66 kDa) CATs detected in *Candida tropicalis*, suggesting that the two proteins localized in different organelles were the products of a single gene. Furthermore, based on the analysis of the N-terminal amino acid sequences

of purified peroxisomal and mitochondrial CATs, the author could propose that these isozymes arose from the difference in the initiation sites of translation. A 71 kDa precursor of mitochondrial CAT, initiating at the first Met, was supposed to be processed to the mature size (66 kDa) after translocation into mitochondria.

Chapter 2 summarizes the results that the genes corresponding to the supposed peroxisomal and mitochondrial CATs, which were truncated from the *Candida tropicalis* CAT gene, were individually expressed in *Saccharomyces cerevisiae*, using the *Candida tropicalis* isocitrate lyase promoter. When 71 kDa CAT was expressed, a 66 kDa protein could be detected, along with the 71 kDa one. The results strongly suggested that the mature size mitochondrial CAT (66 kDa) was formed by processing of the 71 kDa protein, but was not a processed or degradated product of the 68 kDa peroxisomal CAT. The CAT protein synthesized was immunocytochemically detected in mitochondria. When 68 kDa CAT, initiating at the second Met (residue No. 19) was expressed, on the other hand, only the 68 kDa protein was observed and CAT immunoreactivity was detected in peroxisomes and cytosol, but not in mitochondria. From these results, it could be demonstrated that mitochondrial and peroxisomal CATs arose from differences in the initiation sites of translation.

Chaper 3 deals with the expression mechanism of CAT isozymes.

The 5' ends of transcripts of *Candida tropicalis* CAT was investigated using the methods of primer extension and RNase protection assay. All 5'ends of transcripts appeared upstream of the first AUG codon, suggesting that peroxisomal CAT, initating at the second AUG codon, was synthesized by a translational readthrough of the first AUG codon. The author also tried to define factors that influence the efficiency of this alternative translation. Various mutated *Candida tropicalis* CAT genes were constructed and expressed in *Saccharomyces cerevisiae*. The results indicated that the structure and sequence context of the region from the 5' end to the second AUG codon were prerequisite for the alternative translation initiation. This phenomena is very rare not only in yeast, but also in higher eukaryotic cells.

Part I Mitochondrial and peroxisomal NADP-linked isocitrate dehydrogenases in *Candida tropicalis* encoded by distinct genes

Chapter 1 Analysis of the gene encoding mitochondrial NADP-linked isocitrate dehydrogenase and characterization of the expressed product

INTRODUCTION

Isocitrate dehydrogenase (IDH) catalyzes the oxidative decarboxylation of isocitrate to form 2-oxoglutarate, coupled to the reduction of a dinucleotide cofactor. In eukaryotic cells, IDHs are classified as NAD-linked enzyme (NAD-IDH) present in mitochondria (1) and NADP-linked enzyme (NADP-IDH) present in cytosol (2) and/or mitochondria (3, 4). NAD-IDH is proposed to be responsible for the isocitrate dehydrogenase activity necessary in the tricarboxylic acid cycle and, subsequently, essential for respiration. Its activity is also essential to supply 2-oxoglutarate, providing an adequate intracellular level of glutamate. In the yeast *Saccharomyces cerevisiae*, NAD-IDH is present in mitochondria (5), while one isozyme of NADP-IDH is localized in cytosol and the other in mitochondria (6,7). It has been shown in *Saccharomyces cerevisiae* that NADP-IDH can also provide 2-oxoglutarate but cannot

compensate for the role of NAD-IDH in the tricarboxylic acid cycle and respiration (8).

In *n*-alkane-assimilating yeast *Candida tropicalis*, the purification and characterization of NADP-linked isocitrate dehydrogenases localized in peroxisomes (Ps-NADP-IDH) were reported by Yamamoto *et al.* (9). The peroxisomal localization of NADP-IDH has not been reported from any other organism hitherto. In order to investigate whether there are further NADP-IDH isozymes, including mitochondrial NADP-IDH (Mt-NADP-IDH) which has not been found in this yeast, and to identify and isolate the NADP-IDH genes, the author has synthesized a DNA probe by PCR using primers containing homologous sequences of NADP-IDHs from various organisms. In this Chapter, the author reports cloning of the gene encoding Mt-NADP-IDH (*CtIDP1*) and the presence of its protein product only in mitochondria of *Candida tropicalis*. The author has also compared the recombinant gene product (CtIDP1) expressed in *Saccharomyces cerevisiae* with peroxisomal NADP-IDH (Ps-NADP-IDH) and discussed the difference and similarity of these isozymes.

MATERIALS AND METHODS

1. Strains and cultivation

Candida tropicalis pK 233 (ATCC 20336) was cultivated aerobically

at 30 ℃ in a medium containing sodium acetate (13.6 g/l) as the sole source of carbon and energy (10). *Escherichia coli* DH-5α [F, *endA*1, *hsdR*17 (r_k^-, m_k^-), *supE*44, *thi*-1, λ^-, *recA*1, *gyrA*96, *ΔlacU*169 (*ϕ80lacZ*ΔM15)] was used as a host cell for cloning and pUC19 as the plasmid for recombination. *Saccharomyces cerevisiae* strain MT8-1 (*Mata ade2 his3 leu2 trp1 ura3*) (11) was used as a host for the expression of the constructed plasmids.

2. Preparation of probe

Primers to isolate the genomic *NADP-IDH* genes (*CtIDPs*) were prepared based on other NADP-IDH sequences and codon usage in *Candida tropicalis* (12). The peptide sequences with high homology, His-Ala-His-Gly-Asp-Gln-Tyr-Lys and Glu-Ala-Ala-His-Glu-Thr-Val-Thr (see Fig. 3), yielded the oligonucleotide primers 5'-CA(C/T)GC(C/T)CA(C/T)GG(C/T) GA(C/T)CA(A/G)TA(C/T)AA(G/A)GC-3' and the oppositely oriented 5'-GT(A/G)AC(A/G)GT(A/G)CC(A/G)TG(A/G)GC(A/G)GC(T/C)TC-3'. A DNA fragment (530bp) was obtained by PCR using these primers and *Candida tropicalis* genomic DNA as the template. This fragment was labeled with biotin-11-dUTP (Bethesda Research Laboratories Life Technologies (BRL), Gaithersburg, MD, U.S.A.) by nick translation and used as a probe. Detection was performed either by a color development reaction with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate or by a luminescence reaction with a Photo Gene Nucleic Acid Detection System Kit (BRL).

3. Screening of genomic *CtIDP* clones

A λEMBL 3 genomic DNA library of *Candida tropicalis* cells was prepared with the genomic DNA isolated from the yeast (13). The clones were screened according to the method of Benton and Davis (14)

4. Restriction enzyme mapping and DNA sequencing

Restriction endonucleases were purchases from Toyobo (Osaka, Japan) and BRL. Reactions were performed under the conditions recommended by the vendors. The DNA fragments were prepared using endonucleases and the Kilo-Sequence deletion kit (Takara Shuzo, Kyoto, Japan). The respective DNA fragments subcloned into pUC19 were analyzed by the dideoxy-chain-termination method (15) using the universal 17-nucleotide primer, the reverse 17-nucleotide primer (Pharmacia, Uppsala, Sweden), and oligonucleotide primers synthesized by a 381A DNA synthesizer (Applied Biosystem, Foster City, CA, U.S.A.).

5. Construction of the plasmid pWIDP1 to express *CtIDP1*

To construct the plasmid pWIDP1 for the high-level expression of *CtIDP1* isolated from *Candida tropicalis*, pWI3 having *UPR-ICL* and *TERM-ICL* (16) was used. PCR was performed to introduce the *CtIDP1* gene into the multicloning sites on pWI3. *CtIDP1* was used as a template, and the primer No.1, 5'-CAACGGATCCCCAGAATGATCAGAG-3' to introduce *BamH*I site which hybridized near the initiation site and the primer No.2, 5'-ATACGCTCGAGTTTACTTCTTCAATCTGTC-3' to introduce *Xho*I site

which hybridized near the termination site were used. *Saccharomyces cerevisiae* MT8-1 cells were transformed with this plasmid and pMW1 as the control plasmid, using the electroporation method (17), respectively.

6. Purification of CtIDP1 expressed in *Saccharomyces cerevisiae*

Saccharomyces cerevisiae (100 g wet cell) grown on SA medium (0.67 % yeast nitrogen base without amino acids, 0.002 % adenine sulfate, 0.002 % L-histidine-HCl, 0.002 % uracil, and 0.003 % L-leucine with 1% sodium acetate) for 30 h at 30 ℃ was suspended in 50 mM potassium phosphate buffer (pH 6.5) containing 10 % glycerol (buffer A) and sonicated for 1 min at 0 ℃ for disintegration. This suspension was centrifuged at 127,000 x *g* for 1 h at 0 °C, and the resulting supernatant was used as the cell-free extract. This cell-free extract was applied to Q-Sepharose FF column (Pharmacia) (2.2 x 15 cm), washed with buffer A and CtIDP1 was eluted by a linear concentration gradient prepared from 200 ml of buffer A and 200 ml of buffer A containing 1 M KCl. Fractions with NADP-IDH activity were collected and concentrated with the Centriplus membrane (Amicon, Beverly, MS, U.S.A.). The final concentration of 2 M ammonium sulfate was added to the concentrated enzyme solution and applied to Phenyl-TOYOPEARL column (2.2 X 22 cm) (TOSOH, Tokyo, Japan) equilibrated and washed with buffer A containing 2 M ammonium sulfate, and CtIDP1 was eluted by linear concentration gradient prepared from 200 ml of buffer A containing 2 M ammonium sulfate and 200 ml of buffer A. The active fractions were

collected, concentrated, and desalted by the Centriplus membrane. This solution was used as the purified enzyme. All operations were performed at 4 ℃.

7. Subcellular fractionation

Candida tropicalis protoplasts prepared from acetate-grown cells were homogenized with a Teflon homogenizer and fractionated by differential centrifugation (18). The particulate fraction (P_2) containing peroxisomes and mitochondria obtained after centrifugation at 20, 000 x *g* was further centifuged at 49,600 x *g* for 2 h in a discontinuous sucrose density gradient (30, 40, 41.3, 42.5 and 50 % (W/V) surose (each 2.5 ml)). The fraction containing catalase was designated as the peroxisomal fraction and that containing cytochrome *c* oxidase as the mitochondrial fraction (19).

8. Partial purification of Mt-NADP-IDH from *Candida tropicalis*

The mitochondrial fraction (fraction 2) from *Candida tropicalis* cells (10 g wet cell) grown on acetate for 10 h was suspended in buffer A and sonicated for 1 min at 0 °C to disintegrate mitochondria. This suspension was centrifuged at 127,000 x *g* for 1 h at 0 °C. The resulting supernatant named the F_2S fraction was applied to Q-Sepharose FF column (2.2 x 15 cm) , the column being washed with buffer A, and Mt-NADP-IDH was eluted by linear concentration gradient prepared from 200 ml of buffer A and 200 ml of buffer A containing 1 M KCl. Fractions with NADP-IDH activity were collected and concentrated with the Centriplus membrane. This solution was used as the partially purified enzyme. All operations were performed at 4 °C.

9. Electrophoresis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Ueda *et al.* (20).

10. Assay of the enzymes and protein

Catalase, cytochrome *c* oxidase, and NADP-IDH were assayed by the methods described by Fukui *et al.* (21) and Nabeshima *et al.* (22). Protein was quantified by the Lowry method (23).

11. Western blot analysis

A polyclonal antiserum was prepared by injection to rabbits with purified CtIDP1 (Sawady Technology, Tokyo, Japan). Western blot analysis was conducted as described by Ueda *et al.* (20) using this antiserum.

12. Immunochemical analysis

Immunochemical titration was carried out using anti-CtIDP1 antiserum (24). The reaction mixture (500 μl) containing the subcellular fraction (100 μl), the antiserum (10 μl), and 50 mM potassium phosphate buffer (pH 7.2) (390 μl) was incubated at 4 °C for 3h and the antigen-antibody precipitate was removed by centrifugation at 10,000 x *g* for 15 min at 0 °C. NADP-IDH activity in the supernatant solution was measured.

13. N-terminal amino acid sequence analysis

After SDS-PAGE, proteins were transferred to a sheet of ProBlott (Applied Biosystems). The filter after transfer was set in a protein sequencer 610A (Applied Biosystems) and analyzed as recommended by the vendor.

14. Northern blot analysis

Total RNA was isolated as described by Kaiser *et al.* (25). Northern blot analysis was carried out as described by Hikida *et al.* (26), using the biotin-labeled *Kpn*I and *Kpn*I genomic DNA fragment in the *CtIDP1* gene. Detection was carried out by the luminescence reaction as described above.

RESULTS AND DISCUSSION

1. Isolation and analysis of a genomic *NADP-IDH* gene from *Candida tropicalis* (*CtIDP1*)

In order to obtain genes encoding NADP-IDH in *Candida tropicalis*, the author constructed primers at locations where amino acid sequences were highly conserved among previously reported NADP-IDHs (see MATERIALS AND METHODS). By PCR, only one DNA fragment (530 bp) was obtained as a candidate for *NADP-IDH* gene fragment, showing a high similarity with the nucleotide sequence of other known NADP-IDHs. Using this DNA fragment as a probe, a gene encoding NADP-IDH (*CtIDP1*) was isolated from the λEMBL 3 genomic DNA library of *Candida tropicalis*. A DNA fragment containing *CtIDP1* was subcloned into pUC19 and named pIDP1. Fig. 1A shows the restriction map of the DNA insert in pIDP1 with the sequence strategy. The *CtIDP1* gene had an open reading frame consisting of 1,290 bp, corresponding to 430 amino acid residues (Fig. 1B). Fig. 2 summarizes

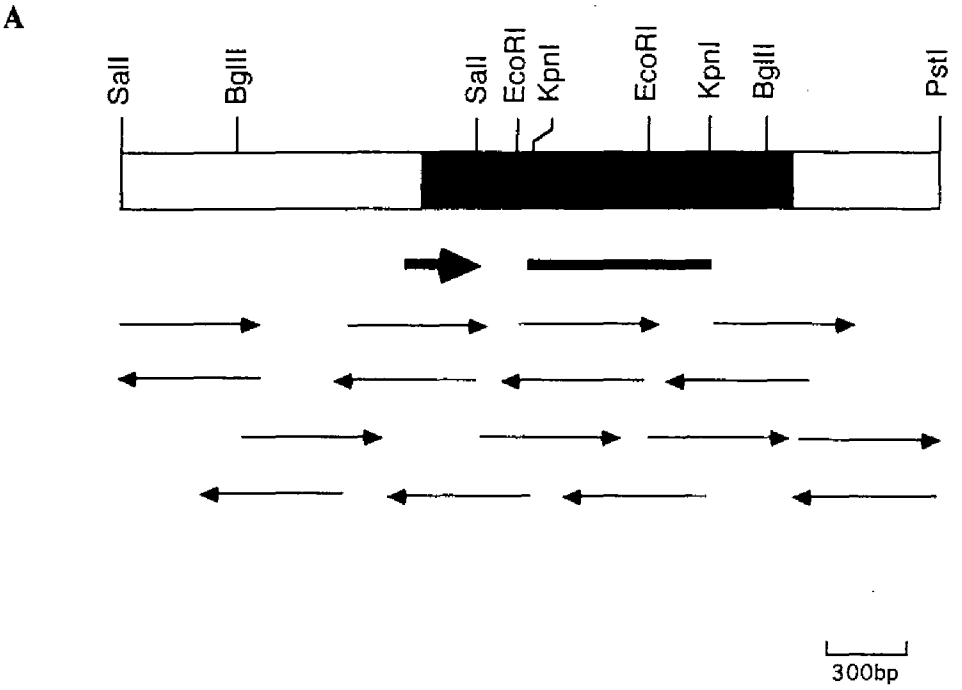
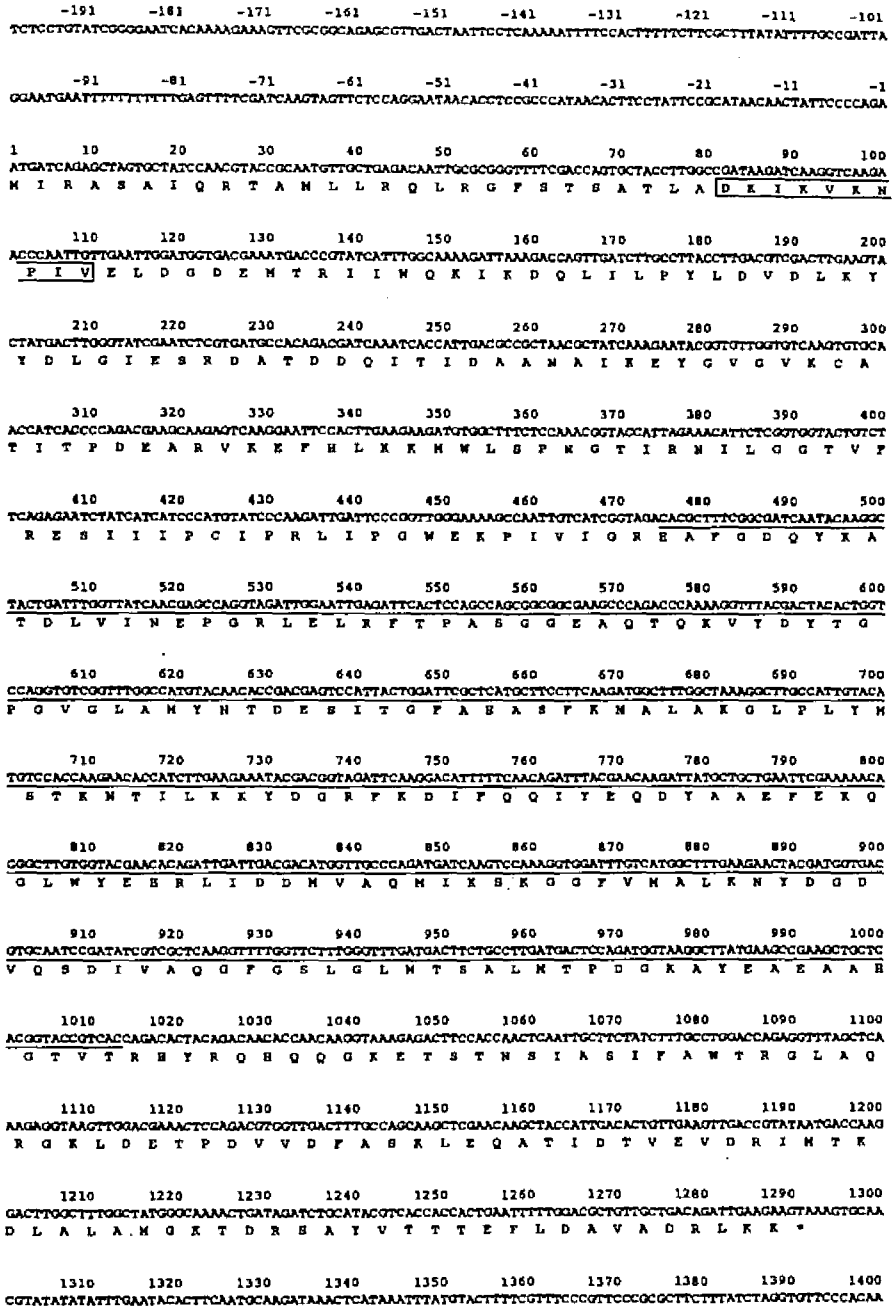


Fig. 1. Restriction enzyme map and sequence strategy of genomic DNA fragment containing the *Candida tropicalis CtIDP1* gene (A) and nucleotide sequence of *Candida tropicalis CtIDP1* gene (B).

A The thick arrow indicates the direction of transcription and the thin arrows indicate the direction and extent of sequence determination. The thick bar corresponds to the DNA fragment used as the probe. The closed box indicates the coding region. **B** The amino acid sequence was deduced from the nucleotide sequence. Position 1 corresponds to the first nucleotide of the ATG initiation codon. The boxed amino acids were identical to the results of N-terminal amino acid sequencing of the partially purified protein. The underlined nucleotide sequence indicates the region identical to the isolated DNA by PCR.

B



the alignment of the amino acid sequences of CtIDP1 and NADP-IDHs from other sources. The similarity of amino acid sequence of CtIDP1 to other NADP-IDHs was as follows; *Saccharomyces cerevisiae* IDP1 (mitochondrial) and IDP2 (cytosolic) (6, 7) 73.1 % and 67.0 %, bovine Mt-NADP-IDH (27) 61.6 %, and pig Mt-NADP-IDH (3) 62.0 %.

2. Expression of *CtIDP1* using *UPR-ICL* in *Saccharomyces cerevisiae*

To prepare an adequate amount of CtIDP1 for further characterization of the enzyme, *CtIDP1* was overexpressed in *Saccharomyces cerevisiae* using the *UPR-ICL* promoter (28). The coding region of *CtIDP1* was inserted into the expression vector pWI3 (16). In cells harboring this plasmid, pWIDP1, the intracellular NADP-IDH activity significantly increased (21.7-fold), compared to that of the control cells containing pMW1. The transformant cells harboring pWIDP1 produced recombinant CtIDP1 with a molecular mass of 44 kDa, indicated by an arrow in Fig.3A, lane 2. The molecular mass of the purified recombinant CtIDP1 (Table 1) was distinct from that observed for Ps-NADP-IDH (45 kDa) purified from *n*-alkane-grown *Candida tropicalis* cells (Fig.3B, lanes 1-3). The N-terminal amino acid sequence of the recombinant CtIDP1 subunit was "Asp-Lys-Ile-Lys-Val-Lys-Asn-Pro-Ile-Val-", which was identical to the amino acid sequence deduced from the gene starting from the residue, Asp-28. No acidic residue was observed in the region processed, showing a characteristic for mitochondrial signal sequences. The results suggested that *CtIDP1* encoded the precursor for

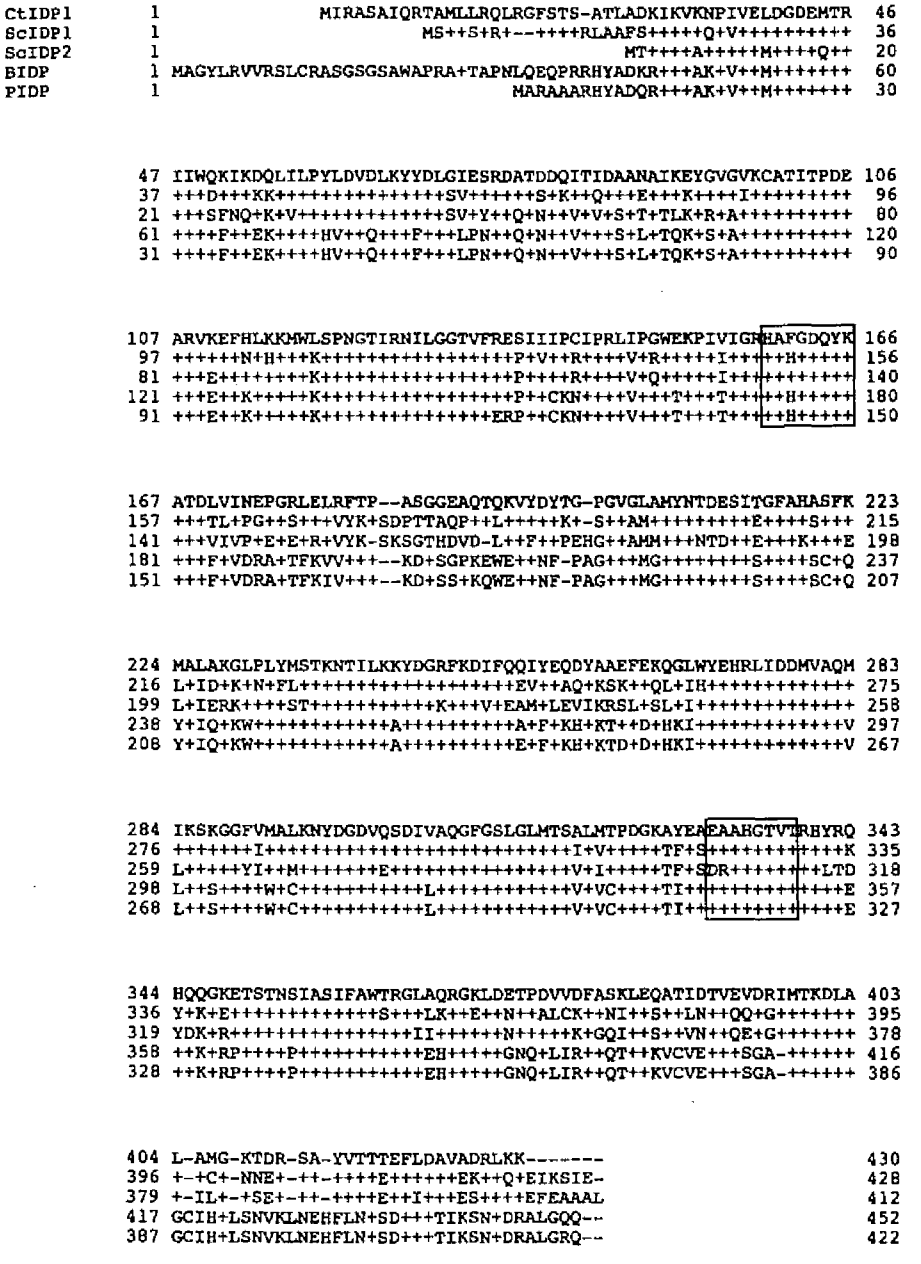
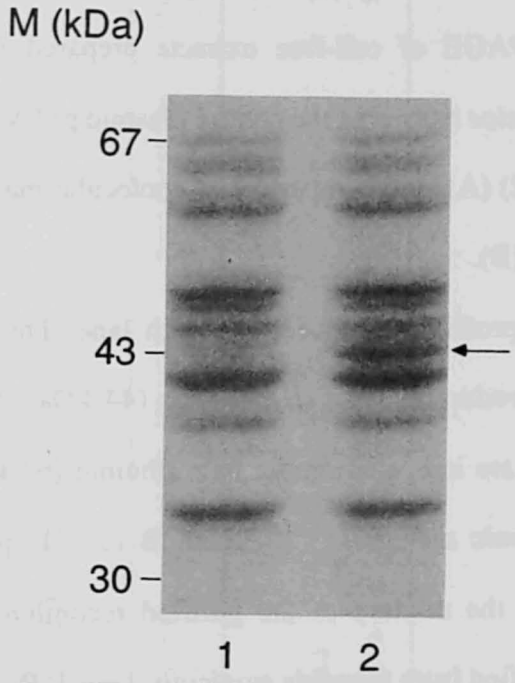

CtIDP1 1 MIRASAIQRTAMLLRQLRGFSTS-ATLADKIKVKNPIVELDGDEMTR 46
ScIDP1 1 MS++S+R+--++++RLAAFS+++++Q+V+++++++++ 36
ScIDP2 1 MT++++A+++++M++++Q++ 20
BIDP 1 MAGYLRVVRSLCRASGSGSAWAPRA+TAPNLQEQPRRHYADKR+++AK+V++M+++++++ 60
PIDP 1 MARAAARHYADQR+++AK+V++M+++++++ 30
47 IIWQKIKDQLILPYLDVDLKYYDLGIESRDATDDQITIDAANAIKEYGVGVKCATITPDE 106
37 +++D+++KK++++++++++++++SV++++++S+K++Q+++E+++K++++I+++++++++ 96
21 +++SFNQ+K+V++++++++++++SV+Y++Q+N++V+V+S+T+TLK+R+A++++++++++ 80
61 ++++F++EK++++HV++Q+++F+++LPN++Q+N++V+++S+L+TQK+S+A++++++++++ 120
31 ++++F++EK++++HV++Q+++F+++LPN++Q+N++V+++S+L+TQK+S+A++++++++++ 90
107 ARVKEFHLKKMWLSPNGTIRNILGGTVFRESIIIPCIPRLIPGWEKPIVIGRHAFGDQYK 166
97 ++++++N+H+++K+++++++++++++++++P+V++R++++V+R++++I+++++H++++++ 156
81 +++E++++++++K+++++++++++++++++P++++R++++V+Q+++++I+++++++++++ 140
121 +++E++K+++++K+++++++++++++++++P++CKN++++V+++T+++T+++++H++++++ 180
91 +++E++K+++++K+++++++++++++++ERP++CKN++++V+++T+++T+++++H++++++ 150
167 ATDLVINEPGRLELRFTP--ASGGEAQTQKVYDYTG-PGVGLAMYNTDESITGFAHASFK 223
157 +++TL+PG++S+++VYK+SDPTTAQP++L+++++K+-S++AM+++++++++E++++S+++ 215
141 +++VIVP+E+E+R+VYK-SKSGTHDVD-L++F++PEHG++AMM+++NTD++E+++K+++E 198
181 +++F+VDRA+TFKVV+++--KD+SGPKEWE++NF-PAG+++MG++++++++S++++SC+Q 237
151 +++F+VDRA+TFKIV+++--KD+SS+KQWE++NF-PAG+++MG++++++++S++++SC+Q 207
224 MALAKGLPLYMSTKNTILKKYDGRFKDIFQQIYEQDYAAEFEKQGLWYEHRLIDDMVAQM 283
216 L+ID+K+N+FL++++++++++++++++++++EV++AQ+KSK++QL+IH++++++++++++ 275
199 L+IERK++++ST++++++++++K+++V+EAM+LEVIKRSL+SL+I++++++++++++++ 258
238 Y+IQ+KW++++++++++++A++++++++++A+F+KH+KT++D+HKI++++++++++++++V 297
208 Y+IQ+KW++++++++++++A++++++++++E+F+KH+KTD+D+HKI++++++++++++++V 267
284 IKSKGGFVMALKNYDGDVQSDIVAQGFGSLGLMTSALMTPDGKAYEAEAAHGTVTRHYRQ 343
276 +++++++I++++++++++++++++++++++++++++I+V+++++TF+S++++++++++++K 335
259 L+++++YI++M++++++++E++++++++++++++++V+I+++++TF+SDR++++++++LTD 318
298 L++S++++W+C++++++++++L++++++++++++V+VC++++TI++++++++++++++E 357
268 L++S++++W+C++++++++++L++++++++++++V+VC++++TI++++++++++++++E 327
344 HQQGKETSTNSIASIFAWTRGLAQRGKLDETPDVVDFASKLEQATIDTVEVDRIMTKDLA 403
336 Y+K+E++++++++++++++S+++LK++E++N++ALCK++NI++S++LN++QQ+G+++++++ 395
319 YDK+R++++++++++++++++II++++++N+++++K+GQI++S++VN++QE+G+++++++ 378
358 ++K+RP++++P++++++++++EH+++++GNQ+LIR++QT++KVCVE+++SGA-++++++ 416
328 ++K+RP++++P++++++++++EH+++++GNQ+LIR++QT++KVCVE+++SGA-++++++ 386
404 L-AMG-KTDR-SA-YVTTTEFLDAVADRLKK-------- 430
396 +-+C+-NNE+-++-++++E++++++EK++Q+EIKSIE- 428
379 +-IL+-+SE+-++-++++E++I+++ES++++EFEAAAL 412
417 GCIH+LSNVKLNEHFLN+SD+++TIKSN+DRALGQQ-- 452
387 GCIH+LSNVKLNEHFLN+SD+++TIKSN+DRALGRQ-- 422

Fig. 2. Alignment of the amino acid sequences of NADP-IDHs from *Candida tropicalis* (CtIDP1), *Saccharomyces cerevisiae* (ScIDP1, ScIDP2), bovine (BIDP), and pig (PIDP).

All sequences are shown by the one-letter amino acid notation. Identical residues to the *Candida tropicalis* NADP-IDH sequence with others are indicated with +. The primer regions for PCR highly conserved among NADP-IDHs are boxed. Gaps have been inserted to achieve the maximum similarity. The amino acids of each enzyme are numbered at its side.

A



B

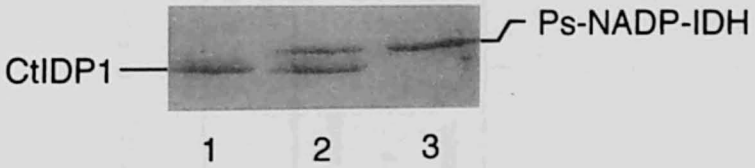


Fig. 3. SDS-PAGE of cell-free extracts prepared from *Saccharomyces cerevisiae* harboring the control plasmid pMW1 (lane 1) or pWIDP1 (lane 2) (A), and comparison of molecular mass profiles of NADP-IDHs (B).

A Forty μg of protein was loaded on each lane. The arrow indicates the overexpressed product of the *CtIDP1* gene (44 kDa). Molecular masses of marker proteins are indicated on the left: albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa). **B** lane 1, purified recombinant CtIDP1; lane 2, the mixture of the purified recombinant CtIDP1 and Ps-NADP-IDH purified from *Candida tropicalis*, lane 3, Ps-NADP-IDH purified from *Candida tropicalis*. Five μg of protein was loaded on each lane.

Table 1 Summary of Purification Procedure of Recombinant CtIDP1.

Fraction	Total Activity (μmol · min^{-1})	Total Protein (mg)	Specific Activity (μmol · min^{-1} · mg^{-1})	Purification (-fold)	Yield (%)
Cell-free Extracts	324	338	0.958	1	100
Q-Sepharose FF	231	14.3	16.1	16.8	71
Phenyl-TOYOPEARL	163	6.78	24.1	25.2	50

Mt-NADP-IDH and was processed after Ala-27 during translocation to mitochondria.

3. Localization of NADP-IDH corresponding to CtIDP1 in *Candida tropicalis*

N-Terminal amino acid sequence of recombinant CtIDP1 suggested its localization in mitochondria. However, in *n*-alkane-grown *Candida tropicalis*, only Ps-NADP-IDH has been found but not Mt-NADP-IDH (9). As the presence of NADP-IDH in mitochondria and/or cytosol was reported in various organisms, the author tried to detect the presence of Mt-NADP-IDH in *Candida tropicalis*. Subcellular fractionation was carried out with cells grown on acetate (Fig. 4), because expression of Ps-NADP-IDH was repressed and peroxisomal proliferation was low in these cells. Cytochrome *c* oxidase, a marker enzyme of mitochondria, was detected predominantly in fraction 2 and catalase, a marker enzyme of peroxisomes, mainly in fraction 5 after the discontinuous sucrose density gradient centrifugation of the particulate fraction (P_2 fraction) of the cells. Under these conditions, a low but distinct NADP-IDH activity was revealed in the mitochondrial fraction (fraction 2) as well as in peroxisomal fraction (fraction 5). To discriminate these NADP-IDHs of *Candida tropicalis*, Western blot analysis was conducted to the mitochondrial and peroxisomal fractions of *Candida tropicalis* with the polyclonal antibodies against the purified recombinant CtIDP1 (Fig. 5). The results revealed that the *CtIDP1* gene product, CtIDP1 (44 kDa), was localized solely in the

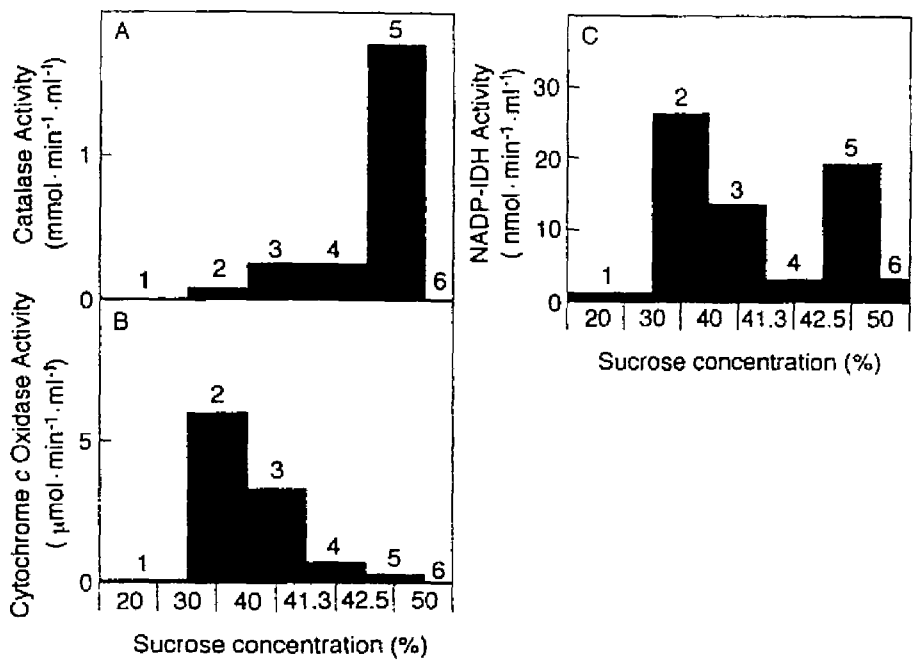


Fig. 4. Particulate localization of enzymes in P_2 fraction from acetate-grown *Candida tropicalis*.

Sucrose density centrifugation of P_2 fraction was carried out as in MATERIALS AND METHODS. The volume of each fraction was as follows: 1, 3.75 ml; 2-5, 2.5 ml each; 6, 1.25 ml. **A**, catalase; **B**, cytochrome *c* oxidase; **C**, NADP-IDH.

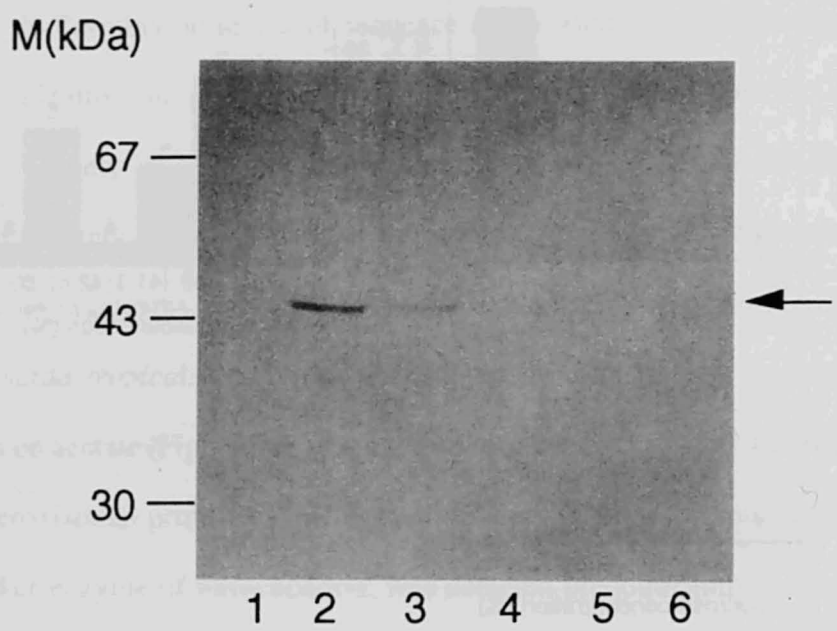


Fig. 5. Western blot analysis of NADP-IDH in particulate fractions of acetate-grown *Candida tropicalis* with anti-CtIDP1 antiserum.

Lanes 1 to 6 correspond to fractions 1 to 6 in Fig. 4. Mitochondrial and peroxisomal fractions were fraction 2 and fraction 5, respectively. Amount of protein loaded for SDS-PAGE was 5 μl of each fraction. An arrow represents the CtIDP1 subunit.

mitochondrial fraction (Fig. 5, fraction 2). Furthermore, N-terminal amino acid sequence of the enzyme partially purified from acetate-grown *Candida tropicalis* was identical to that of the recombinant CtIDP1. These results gave a conclusive evidence that the *CtIDP1* gene surely encoded Mt-NADP-IDH of *Candida tropicalis*. It is interesting that there was no cross-reaction of the antibodies to Ps-NADP-IDH (fraction 5).

4. Comparison of the properties of Ps- and Mt-NADP-IDH isozymes of *Candida tropicalis*

The specific activity of the purified Mt-NADP-IDH (CtIDP1) was 24.1 $\mu mol.min^{-1}.mg^{-1}$ and the K_m values were 54 μM for DL-isocitrate, 15 μM for NADP, and 104 μM for Mg^{2+}, no significant differences being observed to those of the peroxisomal enzyme (9). Mt-NADP-IDH was also inhibited by 2-oxoglutarate and oxalacetate/glyoxylate, as was the case for Ps-NADP-IDH. The fact that these two enzymes were immunochemically distinct as shown in Fig. 5 was further confirmed by immunochemical titration experiments (Fig. 6). The anti-CtIDP1 antiserum could completely abolish the activity present in the mitochondrial fraction, while only an 11 % decrease was observed in the peroxisomal fraction. The distinct immunochemical property of the two isozymes suggested that Ps-NADP-IDH might be encoded by a different gene, which has not been cloned yet. This differs from the case of carnitine acetyltransferase isozymes of *Candida tropicalis*, localized in

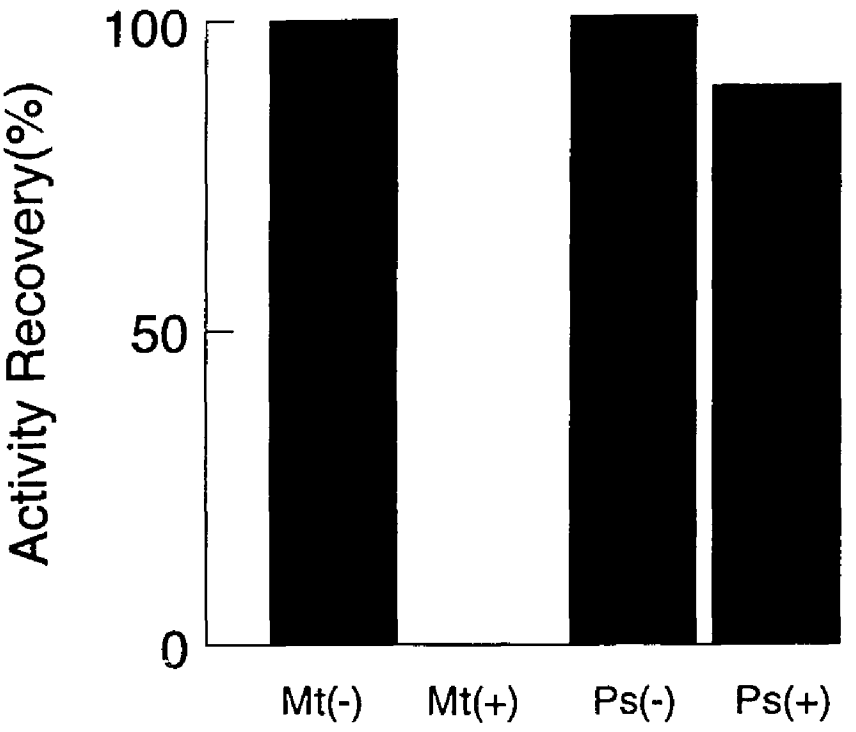


Fig. 6. Immunochemical titration analysis of peroxisomal and mitochondrial NADP-IDHs with anti-CtIDP1 antiserum.

Mitochondrial fraction (fraction 2 in Fig. 4) and peroxisomal fraction (fraction 5 in Fig. 4) were treated with anti-CtIDP1 antiserum (+) or nonimmunized serum (-), respectively, as described in MATERIALS AND METHODS. Each value is the percentage of the remaining activity relative to that after nonimmunized serum treatment.

peroxisomes and mitochondria, respectively (29). They are encoded by a single gene and their localizations are regulated by distinct translational initiation sites (see Part II, Chapter 1). Therefore, although their molecular masses differ, the two enzymes are immunochemically indistinguishable.

5. Expression levels of *CtIDP1* in *Candida tropicalis* in response to carbon sources

It is of interest to know whether gene expression of *CtIDP1* is inducible or constitutive in *Candida tropicalis*. The levels of the *CtIDP1* gene products (Mt-NADP-IDH) and RNA transcripts were measured by Western blot and Northern blot analyses with whole cells grown on glucose, acetate or *n*-alkane, respectively. Although a slight decrease was observed in *n*-alkane-grown cells, similar levels of Mt-NADP-IDH could be detected in cells grown on these carbon sources (Fig. 7A). Furthermore, the same tendency was found for *CtIDP1* mRNA in glucose-, acetate-, and *n*-alkane-grown cells (Fig.7B). These results indicated that *CtIDP1* expression is not subject to catabolite repression by glucose, similar to the case of Mt-NADP-IDH gene expression in *Saccharomyces cerevisiae* (30). This is distinctly different from the case of Ps-NADP-IDH (9), which is strongly induced when cells are grown on *n*-alkanes.

In the case of *Saccharomyces cerevisiae*, disruption of either *IDP1* or *IDP2* along with disruption of *IDH2*, a gene encoding the β-subunit of NAD-

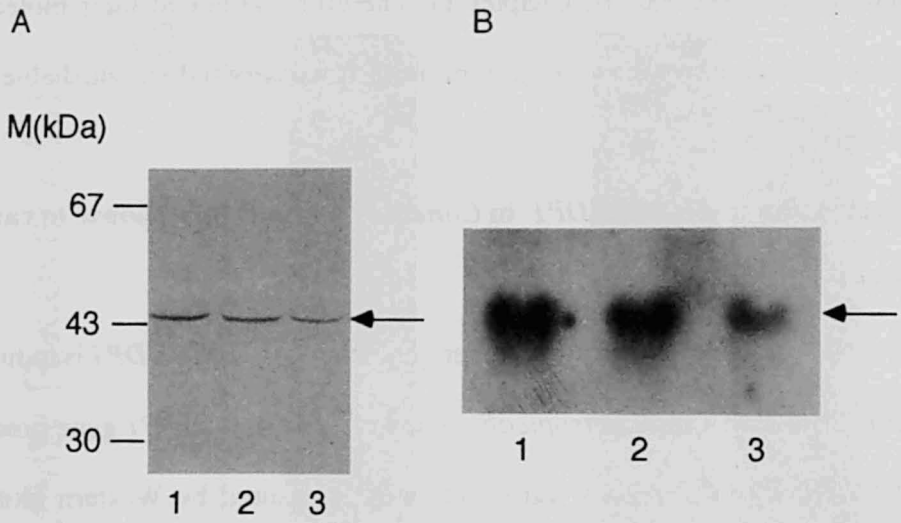


Fig. 7. Western blot analysis of Mt-NADP-IDH (A) and Northern blot analysis of *CtIDP1* mRNA (B) from *Candida tropicalis* cells grown on various carbon sources.

A The respective proteins (150 μg) from glucose- (lane 1), acetate- (lane 2), and *n*-alkane-grown cells (lane 3) were applied to SDS-PAGE. The arrow indicates the Mt-NADP-IDH subunit (44 kDa). Molecular masses of marker proteins are indicated on the left: albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa). **B** Total RNAs (10 μg) isolated from cells grown on glucose (lane 1), acetate (lane 2), and *n*-alkane (lane 3) at the exponential growth phase were separated by electrophoresis and hybridized with the DNA probe for *CtIDP1*. The arrow represents the *CtIDP1* mRNA.

IDH, resulted in a phenotype unable to grow on acetate, giving glutamate auxotrophy. However, in cells where active mitochondrial NAD-IDH was present, disruption of both *IDP* genes did not lead to a significant phenotype. Thus, the precise physiological roles of the two isozymes of NADP-IDH still remain unclear (8). In *Candida tropicalis*, the peroxisomal and mitochondrial localization of NADP-IDH isozymes differs from other organisms and the enzymes may play distinct roles in metabolism or biosynthesis. With the screening method reported here the author has not been able to isolate a gene encoding Ps-NADP-IDH. Isolation of the Ps-NADP-IDH gene and comparison with *CtIDP1* will be described in Chapter 2 of this Part.

SUMMARY

Although peroxisomal localization of NADP-linked isocitrate dehydrogenase (Ps-NADP-IDH) was first demonstrated in *Candida tropicalis*, mitochondrial isozyme (Mt-NADP-IDH) has not been found in this yeast. Here, the author reported the presence of Mt-NADP-IDH in the yeast by screening its gene. In order to isolate genes encoding NADP-IDH, a DNA probe was synthesized by PCR using primers containing homologous sequences of NADP-IDHs from various organisms. One genomic DNA clone was isolated from the yeast λEMBL3 library with this probe. The nucleotide sequence of the gene (*CtIDP1*) revealed a 1,290 bp open reading frame, corresponding to

a 430 amino acid protein with a high similarity to previously reported NADP-IDHs. Overexpression of *CtIDP1* in *Saccharomyces cerevisiae* gave a high intracellular NADP-IDH activity, and the purified recombinant NADP-IDH (CtIDP1) was shown to be a homodimer with subunit molecular mass of approximately 44 kDa, different from that of Ps-NADP-IDH (45 kDa) previously purified from *Candida tropicalis*. N-terminal amino acid sequence analysis of the protein revealed that the first 27 amino acids were processed, suggesting that the gene encoded mitochondrial NADP-IDH. Western blot analysis of the subcellular fractions from acetate-grown *Candida tropicalis* with polyclonal antibodies raised against the recombinant CtIDP1 showed that the CtIDP1 was localized in mitochondria but not in peroxisomes. Similar levels of *CtIDP1* mRNA and its protein product could be detected in cells grown on glucose, acetate, and *n*-alkane, although a slight decrease was observed in *n*-alkane-grown cells. From these results, CtIDP1 was demonstrated to be Mt-NADP-IDH. The properties of Mt-NADP-IDH and Ps-NADP-IDH isozymes were proved to be similar, but they were immunochemically distinct, suggesting the presence of another gene responsible for Ps-NADP-IDH in *Candida tropicalis*.

REFERENCES

1. Ehrlich, R.S. & Colman, R.F. (1990) *Biochemistry* **29**, 5179.

2. Carlier, M.-F. & Pantaloni, D. (1973) *Eur. J. Biochem.* **37**, 341.

3. Haselbeck, R.J., Colman, R.F. & McAlister-Henn, L. (1992) *Biochemistry* **31**, 6219.

4. Macfarlane, N., Mathews, B. & Dalziel, K. (1977) *Eur. J. Biochem.* **74**, 553.

5. Cupp, J.R. & McAlister-Henn, L. (1992) *J. Biol. Chem.* **267**, 16417.

6. Haselbeck, R.J. & McAlister-Henn, L. (1991) *J. Biol. Chem.* **266**, 2339.

7. Loftus, T.M., Hall, L.V., Anderson, S.L. & McAlister-Henn, L. (1994) *Biochemistry* **33**, 9661.

8. Zhao, W.-N. & McAlister-Henn, L. (1996) *Biochemistry* **35**, 7873.

9. Yamamoto, S., Atomi, H., Ueda, M. & Tanaka, A. (1995) *Arch. Microbiol.* **163**, 104.

10. Tanaka, A., Yamamura, M., Kawamoto, S. & Fukui, S. (1977) *Appl. Environ. Microbiol.* **34**, 342.

11. Tajima, M., Nogi, Y. & Fukasawa, T. (1985) *Yeast* **1**, 67.

12. Kurihara, T., Ueda, M., Kanayama, N., Kondo, J., Teranishi, Y. & Tanaka, A. (1992) *Eur. J. Biochem.* **210**, 999.

13. Atomi, H., Ueda, M., Hikida, M., Hishida, T., Teranishi, Y. & Tanaka, A. (1990) *J. Biochem.* **107**, 262.

14. Benton, W.D. & Davis, R.W. (1977) Science **196**, 180.

15. Sanger, F., Nickelen, S. & Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463.

16. Kanai, T., Atomi, A., Umemura, K., Ueno, H., Teranishi, Y., Ueda, M. & Tanaka, A. (1996) *Appl. Microbiol. Biotechnol.* **44**, 759.

17. Brecker, D.M. & Guarente, L. (1991) *Methods Enzymol.* **194**, 182.

18. Kurihara, T., Ueda, M. & Tanaka, A. (1988) *FEBS Lett.* **229**, 215.

19. Ueda, M., Yamanoi, K., Morikawa, T., Okada, H. & Tanaka, A. (1985) *Agric. Biol. Chem.* **49**, 1821.

20. Ueda, M., Mozaffar, S., Atomi, H., Osumi, M. & Tanaka, A. (1989) *J. Ferment. Bioeng.* **68**, 411.

21. Fukui, S., Kawamoto, S., Yasuhara, S., Tanaka, A., Osumi, M. & Imaizumi, F. (1975) *Eur. J. Biochem.* **59**, 561.

22. Nabeshima, S., Ishiyama, S., Tanaka, A. & Fukui, S. (1977) *Agric. Biol. Chem.* **41**, 509.

23. Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) *J. Biol. Chem.* **193**, 265.

24. Ueda, M., Tanaka, A., Horikawa, S., Numa, S. & Fukui, S. (1984) *Eur. J. Biochem.* **138**, 451.

25. Kaiser, C., Michaelis, S. & Mitchell, A. (1994) *Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press.*

26. Hikida, M., Atomi, H., Fukuda, Y., Aoki, A., Hishida, T., Teranishi, Y., Ueda, M. & Tanaka, A. (1991) *J. Biochem.* **110**, 909.

27. Huh, T.-L., Ryu, J.-H., Huh, J-.W., Sung, H.-C., Oh, I.-U., Song, B.J. & Veech, R.L. (1993) *Biochem. J.* **292**, 705.

28. Umemura, K., Atomi, H., Kanai, T., Teranishi, Y., Ueda, M. & Tanaka, A. (1995) *Appl. Microbiol. Biotechnol.* **43**, 489.

29. Kawamoto, S., Ueda, M., Nozaki, C., Yamamura, M., Tanaka, A. & Fukui, S. (1978) *FEBS Lett.* **96**, 37.

30. Haselbeck, R.J. & McAlister-Henn, L. (1993) *J. Biol. Chem.* **268**, 12116.

Chapter 2. Gene analysis of a novel NADP-linked isocitrate dehydrogenase localizad in peroxisomes of *Candida tropicalis*

INTRODUCTION

Though only one NADP-linked enzyme (EC 1.1.1.42) is responsible for the oxidative decarboxylation of isocitrate to 2-oxoglutarate in *Escherichia coli* (1, 2), multiple isozymes of IDH, that vary in subcellular localization, regulation, subunit structure, and cofactor specificity, are present in eukaryotic cells. In *n*-alkane-assimilating yeast, *Candida tropicalis*, there are three distinct IDH isozymes. First, the mitochondrial NAD-linked isozyme (EC 1.1.1.41) is an allosteric enzyme believed to catalyze a key regulatory step in the tricarboxylic acid cycle (3). The others are NADP-linked isozymes localized in mitochondria (Chapter 1) and peroxisomes (3). There have been no reports of an NADP-IDH localized in peroxisomes in other organisms as of yet. However, it is notable that the C-terminal sequence of rat cytosolic NADP-linked IDH was "-Ala-Lys-Leu" (4), which represents a proposed peroxisomal targeting signal (5). In the case of *Saccharomyces cerevisiae*, NADP-IDHs have been reported to be only present in cytosol (6) and mitochondria (7). When NAD-IDH was functional, no dramatic differences in growth were detected for mutant strains lacking either or both NADP-IDH isozymes in *Saccharomyces cerevisiae* (8). Although they presumably provide 2-

oxoglutarate for endogenous glutamate synthesis or NADPH, their precise metabolic functions are not well understood. In *Candida tropicalis*, the presence of NADP-IDHs in both peroxisomes and mitochondria is attractive in terms of their metabolic functions and relationships. Especially, the physiological roles and molecular evolution of Ps-NADP-IDH is of great interest. In this chapter, the author reports the first case of cloning and sequencing of a peroxisomal NADP-IDH gene (*CtIDP2*). The author compared the primary structure of the Ps-NADP-IDH with previously reported NADP-IDHs from various sources and subcellular compartments. The author also investigated the level of gene expression in response to carbon source.

MATERIALS AND METHODS

1. Strains and vectors

Escherichia coli DH-5α was used as a host cell for cloning and pUC19 as a plasmid for recombination. *Saccharomyces cerevisiae* MT8-1 (9) was used as a host for the expression of *CtIDP2* and pWI3 (10) as a shuttle vector plasmid.

2. Preparation of a probe

Primers to isolate *CtIDP2* were prepared based on the results of N-terminal amino acid sequence analysis, other NADP-IDH sequences, and the codon usage in *Candida tropicalis* pK233 (ATCC 20336) (11). The

oligonucleotides used were 5'-GG(C/T)GA(A/G)AT(C/T)CA(A/G)AA(A/G)AT(C/T)AC-3' and 5'-GT(A/G)AC(A/G)GT(A/G)CC(A/G)TG(A/G)GC(A/G)GC(T/C)TC-3'. A DNA fragment (910 bp) with high identity, but distinct from the Mt-NADP-IDH gene (*CtIDP1*) was obtained by PCR using these primers. In order to construct a probe which would show little cross-hybridization with *CtIDP1*, the author tried to isolate the 5' noncoding region, assuming low identity between *CtIDP1* and *CtIDP2*. The method of the 5'-rapid amplification of cDNA ends (5'-RACE) was adopted and the primer used was 5'-ATTTCAAGATGGCTTCAGCAGCA-3'. 5'-CAAGATGGATCCAGCAGCATCG-3', containing a *BamH*I site and the anchor primer containing an *Sal*I site, 5'- GGCCACGCGCGTCGACTAGTACGGGGGGGGGGGGGGGG-3' were used for amplification. Total RNA containing mRNA was extracted from *n*-alkane-grown *Candida tropicalis* cells as described by Kaiser *et al.* (12). 5'-RACE system kit (Bethesda Research Laboratories Life Technologies (BRL), Gaithersburg, MD, U.S.A.) was used together with 1 μg total RNA extracted and the primers synthesized according to the recommended procedures by the vendor. This cDNA fragment obtained by the 5'-RACE method was labeled with biotin-11-dUTP (BRL) by nick translation and used as a probe. Detection was performed by a luminescence reaction with a Photo Gene Nucleic Acid Detection System Kit (BRL).

3. Screening of genomic *CtIDP2* clones

The clones were screened from a λEMBL 3 genomic DNA library of

Candida tropicalis (13) as described in Chapter 1 (14).

4. Restriction enzyme mapping and DNA sequencing

Restriction enzyme mapping and DNA sequencing were performed as in the preceding chapter (15).

5. Construction of the plasmid pWIDP2 to express *CtIDP2*

λCtIDP2 was used as the template for PCR. *Xho*I site at the 3' end was introduced for the expression construct. The 1.2 kbp fragment was amplified using the oligonucleotides 5'-TACAAACCATGGGCGAAATTCA G-3' and 5'-ATTTACCTCGAGTCTAGTAGCCC-3'. This fragment was digested by *Xho*I and *Nco*I, and then was subcloned into the *Nco*I-*Xho*I site of pWI3, harboring *UPR-ICL* (10). The resulting plasmid, pWIDP2, was transformed to *Saccharomyces cerevisiae* using the electroporation method (16).

6. Cultivation and preparation of cell-free extracts

Saccharomyces cerevisiae bearing the constructed plasmid was cultivated aerobically at 30 °C. After precultivation in YPD medium (1 % yeast extract, 1 % peptone, and 2 % glucose by mass), cells were washed thoroughly with distilled water and transferred to YPA medium (1 % yeast extract, 1 % peptone, and 0.275 % acetate by mass). Cell-free extracts were prepared by disintegrating the cells (0.1-0.25 g dry cells ml^{-1}) by sonication at 20 kHz for 2.5 min at 0 ℃ in 50 mM potassium phosphate buffer (pH 7.2) followed by centrifugation at 10,000 x *g* for 20 min at 4 ℃.

7. Electrophoresis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described in the preceding chapter (17).

8. N-terminal amino acid sequence analysis

N-terminal amino acid sequence were determined as in Chapter 1.

9. Assay of enzymes and protein

NADP-IDH was assayed by the methods described in the preceding chapter (18). Protein was quantified by the modified Lowry method (19).

10. Alignment of amino acid sequences

The multiple alignment of protein sequences and the number of similarity among sequences were obtained with the program ALIGN contained within the ODEN program provided by DNA Databank Japan (DDBJ). The gap of the N- and C-termini was deleted. Amino acid sequences of eukaryotic NADP-IDHs from other sources are deposited in PIR and SwissProt with accession numbers.

11. Construction of phylogenetic tree

The number of amino acid substitutions was estimated with the DISTA program (20). The distance matrix was made with the DMATA program (20). The phylogenetic tree was constructed by the neighbor-joining method with the TREENJ program (21). Bootstrap resampling was performed with the BSTRAP program (22) at least 200 times. These programs were contained within the ODEN program provided by DDBJ.

12. Northern blot hybridization analysis

Northern blot hybridization analysis was carried out as described in the preceding chapter (23), using the biotin-labeled cDNA probe.

RESULTS AND DISCUSSION

1. Isolation and expression of genomic DNA encoding Ps-NADP-IDH

Yamamoto *et al.* (3) have already purified and characterized Ps-NADP-IDH of *n*-alkane-grown *Candida tropicalis* cells. The author analyzed the N-terminal amino acid sequence of Ps-NADP-IDH by the Edman degradation procedure. The amino acid sequence of Ps-NADP-IDH subunit was "Gly-Glu-Ile-Gln-Lys-Ile-Thr-Val-Lys-Asn-Pro-Ile-Val-Glu-Met-". This peptide sequence showed high similarity to previously reported NADP-IDH enzymes from other sources. To prepare cDNA specific to *CtIDP2*, the method of PCR and the 5'-rapid amplification of cDNA ends (5'-RACE) (see MATERIALS AND METHODS) were employed. After the final amplification, a 390 bp cDNA fragment could be isolated. The nucleotide sequence analysis of the cDNA fragment revealed that the deduced amino acid sequence contained the N-terminal amino acid sequence obtained from purified Ps-NADP-IDH. A λEMBL3 genomic DNA library of *Candida tropicalis* was screened for *CtIDP2* with this cDNA probe and a clone named λ*CtIDP2* was isolated. The inserted DNA fragment of λ*CtIDP2* was subcloned into pUC19 and

named pIDP2 (Fig. 1A). The nucleotide sequence of *CtIDP2* and the deduced amino acid sequence are shown in Fig. 1B. The gene contained an open reading frame composed of 1,233 bp, corresponding to 411 amino acid residues. The predicted molecular mass was 46,200 Da, corresponded well to the apparent molecular mass measured by SDS-PAGE (45 kDa). The N-terminal amino acid sequence of Ps-NADP-IDH corresponded to the first 15 amino acid residues after the first methionine deduced from the nucleotide sequence of *CtIDP2*, indicating there was no processing of the protein during its localization to peroxisomes. In the 5'-flanking region, there were two TATA boxes at positions -133 and -238.

This *CtIDP2* was expressed in *Saccharomyces cerevisiae* MT8-1 under the control of the *Candida tropicalis* isocitrate lyase promoter (*UPR-ICL*) (10). The specific NADP-IDH activity of acetate-grown *Saccharomyces cerevisiae* bearing pWIDP2 was 225 times higher than that of the cells containing control vector pMW1. The high expression product was identified by SDS-PAGE (Fig. 2, lane 1, indicated by arrow).

2. Comparison of amino acid sequences for peroxisomal NADP-IDH (CtIDP2) and NADP-IDHs from other sources

The amino acid sequences for CtIDP2 and NADP-IDHs from other sources (4, 6, 7, 24-28) were compared (Fig. 3). Computer alignment revealed that among yeast NADP-IDHs, CtIDP2 possessed 73 % identity with CtIDP1, 71.5 % and 69.6 % with *Saccharomyces cerevisiae* IDP1 (mitochondria)

A

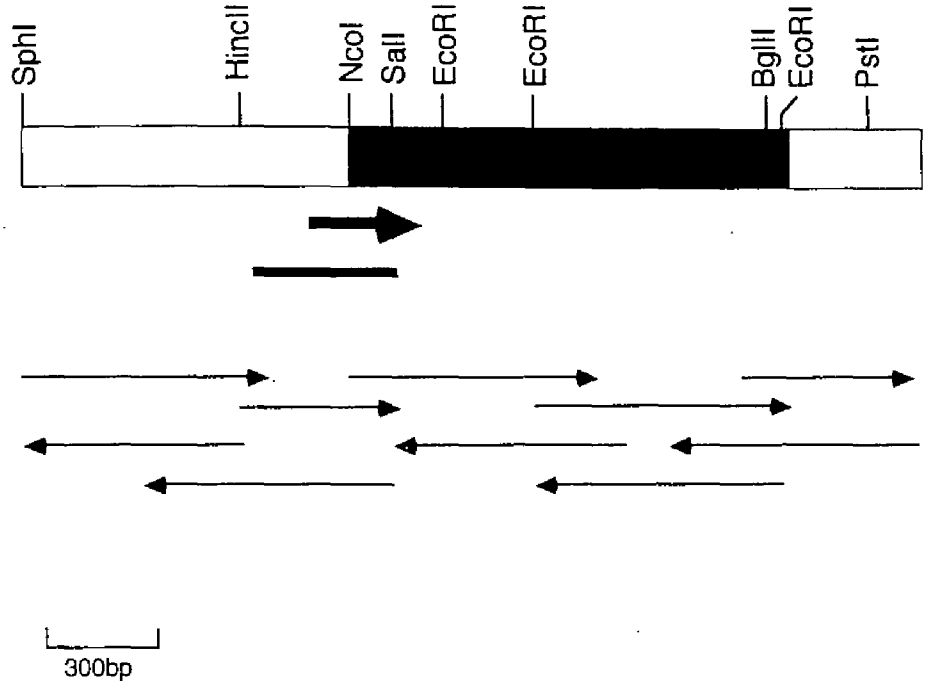
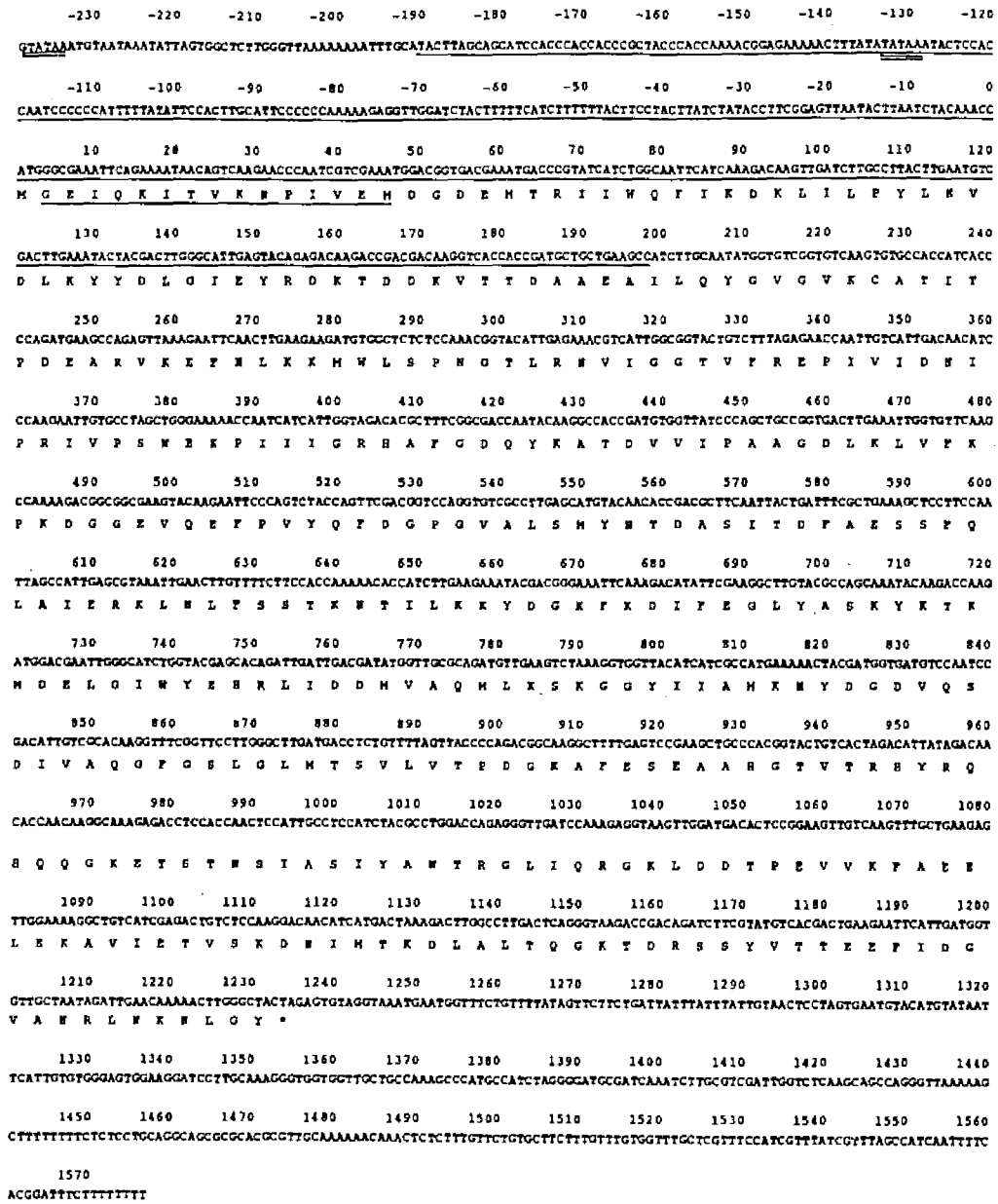


Fig. 1. Restriction map and sequencing strategy of genomic DNA fragment containing Ps-NADP-IDH (*CtIDP2*) (A) and its nucleotide sequence (B).

A The thick arrow indicates the direction of transcription and the thin arrows indicate the direction and extent of sequence determinations. The thick bar corresponds to the cDNA portion. The closed box indicates the coding region. **B** The amino acid sequence was deduced from the nucleotide sequence. Position 1 corresponds to the first nucleotide of the ATG initiation codon. TATA box sequences are indicated by the double underline. The underlined nucleotide sequences were identical to those of the isolated cDNA. The underlined amino acid sequence was the same as the N-terminal amino acid sequence of purified Ps-NADP-IDH.

B



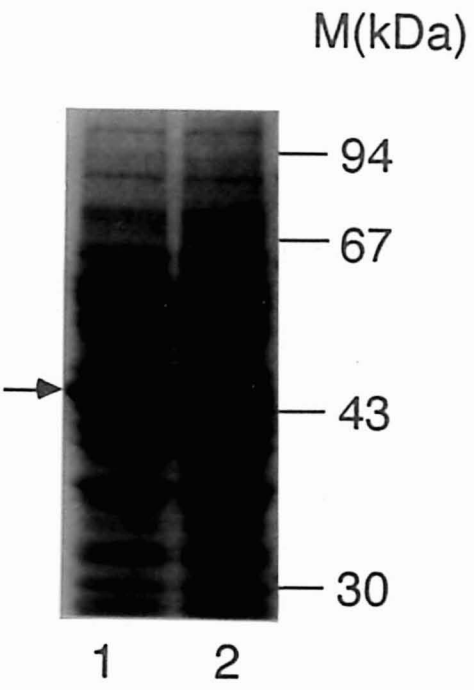
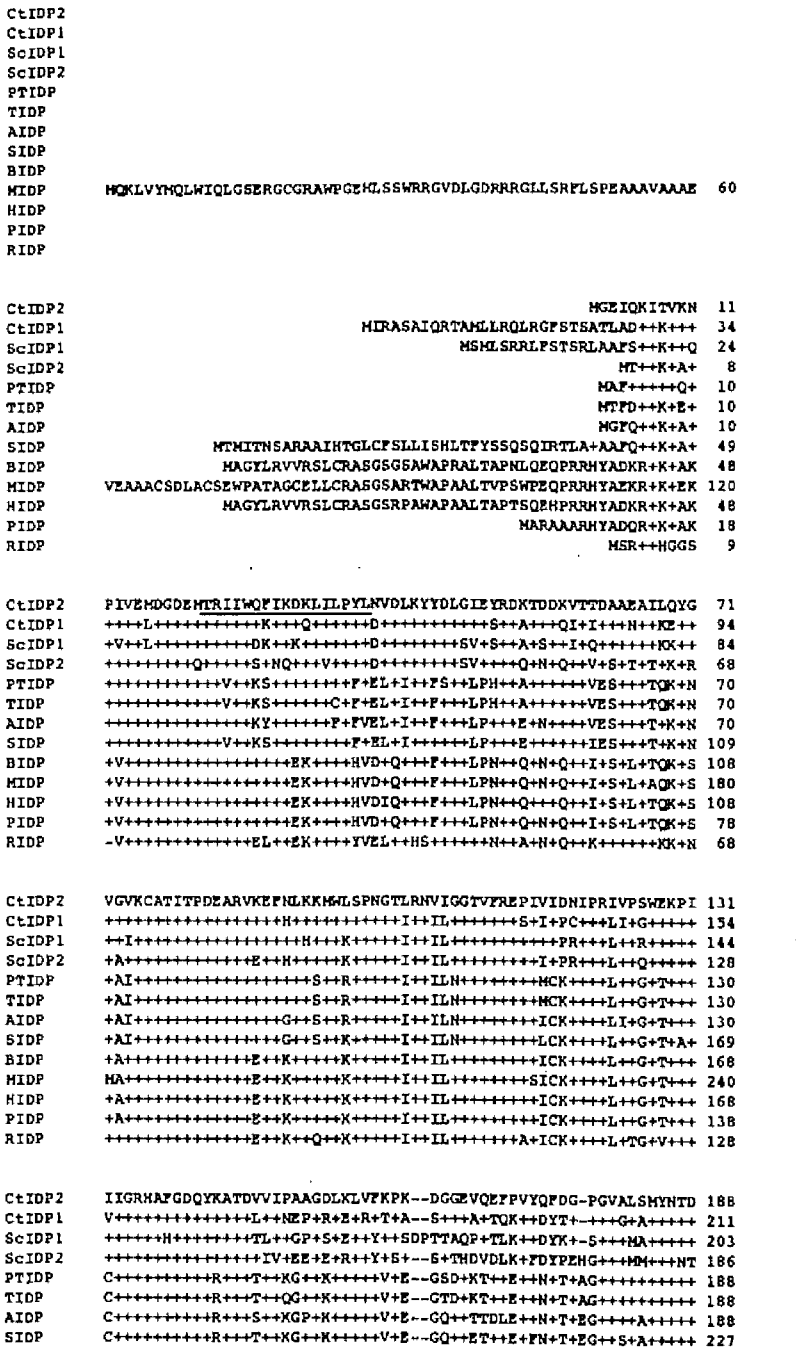


Fig. 2. SDS-PAGE of cell-free extracts prepared from *Saccharomyces cerevisiae* harboring pWIDP2 (lane 1) or control plasmid pMW1 (lane 2).

Fifty μg of protein was loaded in each lane. The arrow indicates the CtIDP2 (45 kDa) band expressed. Molecular masses (M) of marker proteins are shown on the right: phosphoryrase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa).


CtIDP2
CtIDP1
ScIDP1
ScIDP2
PTIDP
TIDP
AIDP
SIDP
BIDP
MIDP
HIDP
PIDP
RIDP
MQKLVYMQLWIQLGSERGCGRAWPGEMLSSWRRGVDLGDRRRGLLSRFLSPEAAAVAAAE 60
MGEIQKITVKN 11
MIRASAIQRTAMLLRQLRGFSTSATLAD++K+++ 34
MSMLSRRLPSTSRLAAFS++K++Q 24
MT++K+A+ 8
MAF+++++Q+ 10
MTFD++K+E+ 10
MGFQ++K+A+ 10
MTMITNSARAAIHTGLCFSLLISHLTFYSSQSQIRTLA+AAFQ++K+A+ 49
MAGYLRVVRSLCRASGSGSAWAPRALTAPNLQEQPRRHYADKR+K+AK 48
VEAAACSDLACSEWPATAGCELLCRASGSARTWAPAALTVPSWPEQPRRHYAEKR+K+EK 120
MAGYLRVVRSLCRASGSRPAWAPAALTAPTSQEHPRRHYADKR+K+AK 48
MARAAAARHYADQR+K+AK 18
MSR++HGGS 9
PIVEMDGDEMTRIIWQFIKDKLILPYLNVDLKYYDLGIEYRDKTDDKVTTDAAEAILQYG 71
+++++L++++++++++K+++Q++++++D++++++++++S++A+++QI+I+++N++KE++ 94
+V++L++++++++++DK++K+++++++D++++++++SV+S++A+S++I+Q++++++KK++ 84
++++++++Q+++++S+NQ+++V++++D++++++++SV++++Q+N+Q++V+S+T+T+K+R 68
++++++++++++V++KS++++++++F+EL+I++FS++LPH++A++++++VES+++TQK+N 70
++++++++++++V++KS++++++C+F+EL+I++F+++LPH++A++++++VES+++TQK+N 70
++++++++++++++KY++++++F+FVEL+I++F+++LP+++E+N+++++VES+++T+K+N 70
++++++++++++V++KS++++++++F+EL+I++++++LP+++E++++++IES+++T+K+N 109
+V+++++++++++++++++EK++++HVD+Q+++F+++LPN++Q+N+Q++I+S+L+TQK+S 108
+V+++++++++++++++++EK++++HVD+Q+++F+++LPN++Q+N+Q++I+S+L+AQK+S 180
+V++++++++++++++++EK++++HVDIQ+++F+++LPN++Q+++Q++I+S+L+TQK+S 108
+V++++++++++++++++EK++++HVD+Q+++F+++LPN++Q+N+Q++I+S+L+TQK+S 78
-V++++++++++++EL++EK++++YVEL++HS++++++N++A+N+Q++K++++++KK+N 68
VGVKCATITPDEARVKEFNLKKMWLSPNGTLRNVIGGTVFREPIVIDNIPRIVPSWEKPI 131
++++++++++++++++++H++++++++++I++IL+++++++S+I+PC+++LI+G+++++ 154
++I+++++++++++++++++H+++K+++++I++IL++++++++++PR+++L++R+++++ 144
+A++++++++++++++E++H+++++K+++++I++IL+++++++++I+PR+++L++Q+++++ 128
+AI++++++++++++++++++S++R+++++I++ILN++++++++HCK++++L++G+T+++ 130
+AI++++++++++++++++++S++R+++++I++ILN++++++++HCK++++L++G+T+++ 130
+AI++++++++++++++++G++S++R+++++I++ILN++++++++ICK++++LI+G+T+++ 130
+AI++++++++++++++++G++S++K+++++I++ILN++++++++LCK++++L++G+T+A+ 169
+A++++++++++++++E++K+++++K+++++I++IL+++++++++ICK++++L++G+T+++ 168
MA++++++++++++++E++K+++++K+++++I++IL++++++++SICK++++L++G+T+++ 240
+A++++++++++++++E++K+++++K+++++I++IL+++++++++ICK++++L++G+T+++ 168
+A++++++++++++++E++K+++++K+++++I++IL+++++++++ICK++++L++G+T+++ 138
++++++++++++++++E++K++Q++K+++++I++IL+++++++A+ICK++++L+TG+V+++ 128
IIGRHAFGDQYKATDVVIPAAGDLKLVFKPK--DGGEVQEFPVYQFDG-PGVALSMYNTD 188
V++++++++++++++L++NEP+R+E+R+T+A--S+++A+TQK++DYT+-+++G+A+++++ 211
++++++H++++++++TL++GP+S+E++Y++SDPTTAQP+TLK++DYK+-S+++MA+++++ 203
++++++++++++++++IV+EE+E+R++Y+S+--S+THDVDLK+FDYPEHG+++MM+++NT 186
C++++++++++R+++T++KG++K+++++V+E--GSD+KT++E++N+T+AG++++++++++ 188
C++++++++++R+++T++QG++K+++++V+E--GTD+KT++E++N+T+AG++++++++++ 188
C++++++++++R+++S++XGP+K+++++V+E--GQ++TTDLE++N+T+EG++++A+++++ 188
C++++++++++R+++T++XG++K+++++V+E--GQ++ET++E+FN+T+EG++S+A+++++ 227
T+++++H++++++++F+VDR++TF+V++T++--++SGPK+WE++N+PA-G++GMG+++++ 225
T+++++H++++++++F+VDR++TF+++++T++--N+SSAK+WE++N+P+-G++GMG+++++ 297
T+++++H++++++++F+ADR++TF+M++T++--++SG+K+WE++N+PA-G++GMG+++++ 225
T+++++H++++++++F+VDR++TF+I++T++--++SSAKQWE++N+PA-G++GMG+++++ 195
I+++++Y++++R+++F+V+GP+KVEITYT++--++SQKVTYL+HD+EEGG+++MG+++Q+ 186

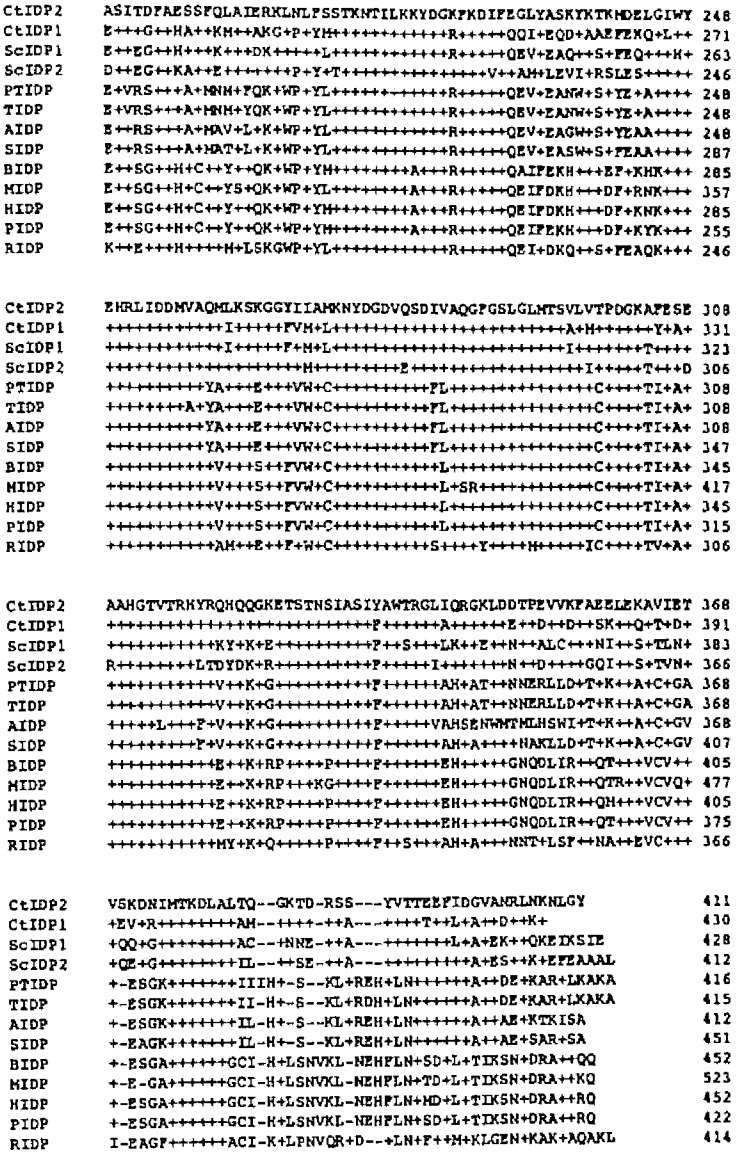


Fig. 3. Alignment of the amino acid sequences of NADP-IDHs from various eukaryotic sources.

All sequences are shown by the one-letter amino acid notation. Identical residues to the CtIDP2 sequence with others are indicated with +. The amino acids of each enzyme are numbered at their sides. The high similality region among CtIDP2, *Saccharomyces cerevisiae* Fox3p, and *Candida tropicalis* Pox4p is indicated by the underline. Abbreviations: PTIDP, potato NADP-linked isocitrate dehydrogenase; TIDP, tabacco NADP-linked isocitrate dehydrogenase; AIDP, alfalfa NADP-linked isocitrate dehydrogenase; SIDP, soybean NADP-linked isocitrate dehydrogenase; BIDP, bovine mitochondrial NADP-linked isocitrate dehydrogenase; MIDP, mouse mitochondrial NADP-linked isocitrate dehydrogenase; HIDP, human mitochondrial NADP-linked isocitrate dehydrogenase; PIDP, pig cytosolic NADP-linked isocitrate dehydrogenase; RIDP, rat cytosolic NADP-linked isocitrate dehydrogenase. Accession numbers in PIR or SwissProt: sp P21954 for ScIDP1, pir A54880 for ScIDP2, pir S47013 for PTIDP, pir S42892 for TIDP, pir S28423 for AIDP, pir S33612 for SIDP, pir S33859 for BIDP, sp P54071 for MIDP, pir S57499 for HIDP, pir M86719 for PIDP, and pir A54756 for RIDP.

and IDP2 (cytosol), respectively. When a phylogenetic tree (Fig. 4) was constructed from all previously reported eukaryotic NADP-IDHs, mammalian mitochondrial NADP-IDHs formed a cluster, as did plant NADP-IDHs. These clusters were shown to be significant after bootstrap analysis (mammalian mitochondrial, 97.5%; plant, 100%). Concerning higher eukaryotes, plant and mammalian NADP-IDHs diverged at an early stage, followed by the divergence of Cy-NADP-IDH and Mt-NADP-IDH in mammalian cells (bootstrap analysis, 80%). The yeast enzymes seemed to all diverge near the origin, but as the branching was too close to the origin of the tree and the bootstrap value was low (26.5%), the linkage among them could not be indicated. However, the divergence of the yeast enzymes from higher eukaryotic enzymes would be the first event which occurred during evolution of eukaryotic NADP-IDHs.

3. Peroxisome targeting signal of CtIDP2

Many studies have shown that peroxisomal matrix proteins contain either one of two well-characterized targeting signals, PTS1 or PTS2. PTS1 consists of a C-terminal sequence "-Ser-Lys-Leu" or its analogues (5, 29). The second peroxisomal targeting sequence, PTS2, has been found on the N-terminus of 3-ketoacyl-CoA thiolase and so on (30, 31), which consists of cleavable or uncleavable sequences of 16 to 26 amino acids in length with a consensus motif of Xn-RL-X_5-HL-Xn. It should be noted, however, that many peroxisomal matrix proteins do not apper to contain either a PTS1 or

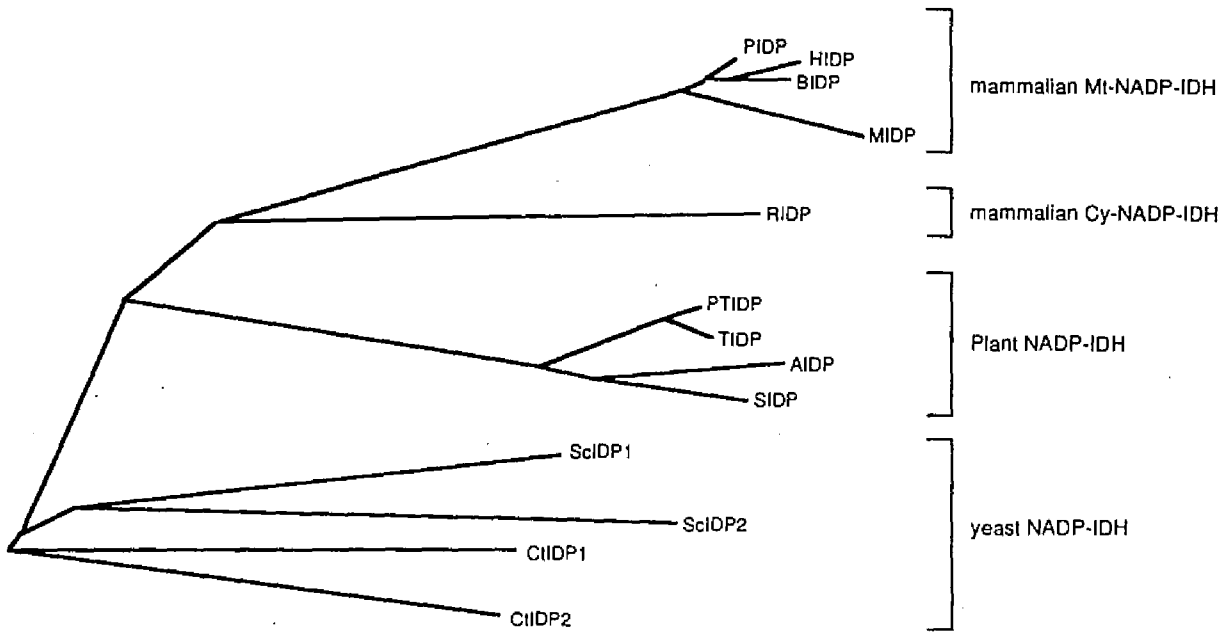


Fig. 4. Phylogenetic relationship of NADP-IDHs from various eukaryotic sources. The phylogenetic tree of NADP-IDHs with all aligned sequences (Fig. 3) was constructed. Abbreviations used are as in Fig. 3.

PTS2 sequence (32). The C-terminal sequence of CtIDP2 was "Leu-Gly-Tyr", which did not satisfy the PTS1 motif. However, near the N-terminus from residue Thr-22 to Leu-38 (region A), a sequence with high similarity with the N-terminus of FOX3, 3-ketoacyl CoA thiolase of *Saccharomyces cerevisiae* (Fig 5A) (33), which is considered as a putative PTS2 peroxisomal signal sequence, was found. Region A also showed similarity with a region in POX4, acyl-CoA oxidase of *Candida tropicalis* (Fig. 5B). This region was included in a fragment (amino acids 309-427 of POX4) which showed peroxisome targeting ability (34). However, the corresponding sequences in other NADP-IDHs localized in cytosol or mitochondria also showed high similarity with region A. It is of interest where the Cy-NADP-IDHs from *Saccharomyces cerevisiae* and rat are localized under the conditions of peroxisomal proliferation, which as of yet have not been investigated. In the case of Cy-NADP-IDH of rat, its peroxisomal localization was further suggested by the C-terminal sequence, "Ala-Lys-Leu", which may be able to serve as a PTS (4). The localization of mitochondrial NADP-IDHs containing putative PTSs could be explained by the fact that mitochondrial signal sequences often override the signals for peroxisome targeting. This was seen in mitochondrial carnitine acetyltransferase of *Candida tropicalis* (see Part II, Chapter 1). Further studies will be needed to elucidate the targeting signal of CtIDP2 to peroxisomes.

A

```
C. tropicalis IDP2          22 TRIIWQFIKDKLILPYL  38
                                ** * *******
S. cerevisiae FOX3           3 QRL--QSIKDHLVLSAM  17
```

B

```
C. tropicalis IDP2          22 TRIIWQFIKDKLILPYL  38
                                * ** *** * *****
C. tropicalis POX4          343 TQLIDYPLHQKRLFPYL 359
```

Fig. 5. The high similality region (region A) between CtIDP2 and *Saccharomyces cerevisiae* FOX3 (A) and *Candida tropicalis* POX4 (B).

The amino acids of each enzyme are numbered at their sides. All sequences are shown by the one-letter amino acid notation. Identical or similar amino acids are indicated by asterisks.

4. Expression levels of *CtIDP2* mRNA in response to carbon source

In yeast, *Saccharomyces cerevisiae*, the level of the cytosolic NADP-IDH (ScIDP2) was elevated with nonfermentable growth conditions, whereas the level of the mitochondrial enzymes (ScIDP1) was constitutive (7). In order to determine the regulation of gene expression of CtIDP2, expression level of *CtIDP2* mRNA was examined by Northern blot analysis when cells were grown on glucose and *n*-alkane (Fig. 6). An increase in the mRNA level was detectable in *n*-alkane-grown cells compared to glucose-grown cells. The author further compared the enzyme level of Ps-NADP-IDH of cells grown on *n*-alkane with that of acetate-grown cells, where glucose repression was released. A 14-fold increase in NADP-IDH activity in the peroxisomal fraction was found in *n*-alkane-grown cells compared to acetate-grown cells. An induction mechanism specific to *n*-alkanes, as is the case of catalase and the β-oxidation enzymes in *Candida tropicalis*, may be present.

While the genes for the mitochondrial (7, 24-26) and cytosolic (4, 6) NADP-IDH isozymes have been cloned and sequenced, this is the first report of cloning and sequence analysis of the peroxisomal isozyme. The peroxisomal isozyme is also the first NADP-IDH whose gene expression is induced by *n*-alkanes and, presumably, other carbon sources that initiate peroxisomal proliferation, *eg.* fatty acids. In the case of *Saccharomyces cerevisiae*, no significant growth phenotype could be attributed to either or both *ScIDP1* and *ScIDP2* disruption (8). However, as the subcellular localization of NADP-

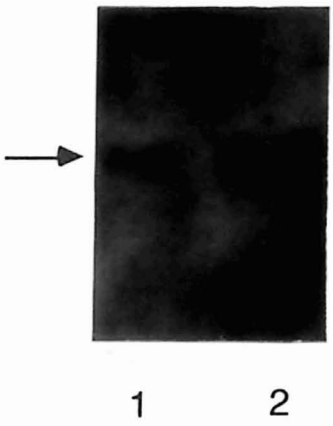


Fig. 6. Northern blot analysis of *CtIDP2* from the cells grown on glucose and *n*-alkane.

Total RNAs (10 μg) isolated from *Candida tropicalis* cultured on glucose (lane 1) and *n*-alkane (lane 2) at the early exponential phase of the growth were separated by electrophoresis and hybridized with the cDNA probe for *CtIDP2*. The arrow indicates the *CtIDP2* mRNA band.

IDHs in *Candida tropicalis* differ from that of *Saccharomyces cerevisiae*, the isozymes in *Candida tropicalis*, especially Ps-NADP-IDH, may serve distinct physiological roles. The participation of Ps-NADP-IDH in *n*-alkane metabolism, glutamate synthesis or NADPH regeneration can be supposed. Further investigations by gene disruption techniques will help to elucidate the functions of NADP-IDH isozymes in *Candida tropicalis*.

SUMMARY

In *n*-alkane-utilizing yeast, *Candida tropicalis*, two NADP-linked isocitrate dehydrogenase (NADP-IDH) isozymes are present, one in mitochondria (Mt-NADP-IDH) and one in peroxisomes (Ps-NADP-IDH). The presence of NADP-IDH in peroxisomes has not been reported in any other organism. In this chapter, the author reported the isolation, sequencing and expression of the gene encoding Ps-NADP-IDH (*CtIDP2*), distinct from Mt-NADP-IDH gene (*CtIDP1*). Based on the N-terminal amino acid sequence of purified Ps-NADP-IDH, a cDNA fragment specific for Ps-NADP-IDH was obtained by the 5'-RACE method. Using this fragment as a probe, the genomic *CtIDP2* gene was isolated. Nucleotide sequence analysis of *CtIDP2* disclosed that the region encoding CtIDP2 had a length of 1,233 bp, corresponding to 411 amino acid residues. The deduced N-terminal amino acid sequence matched the results obtained from the purified protein. When

this *CtIDP2* was expressed in *Saccharomyces cerevisiae* using the *Candida tropicalis* isocitrate lyase gene promoter (*UPR-ICL*), high intracellular NADP-IDH activity was observed. Comparison of amino acid sequences and phylogenetic tree analysis with NADP-IDH enzymes from all reported eukaryotic sources showed that mammalian mitochondrial NADP-IDHs formed a cluster, as did plant NADP-IDHs. CtIDP2 and other yeast NADP-IDHs were not included in these clusters and seemed to diverge at an early stage from all other enzymes of higher eukaryotes. Ps-NADP-IDH had no typical C-terminal peroxisomal targeting signals and no processing could be found at the N-terminus. However, a specific region was found near the N-terminus of the protein with a high similarity with both the putative N-terminal peroxisomal targeting signal sequence of FOX3 of *Saccharomyces cerevisiae* and an internal peroxisomal targeting signal of POX4 of *Candida tropicalis*. The results of Northern blot analysis indicated that the biosynthesis of CtIDP2 was induced in a medium containing alkanes as carbon source, where proliferation of peroxisomes is observed.

REFERENCES

1. Plaut, G.W.E. (1963) *The Enzymes* Vol. 7. *Academic Press, New York*, 105.
2. Ragland, T.E., Kawasaki,T. & Lowenstein, J.M. (1966) *J. Bacteriol.* **91**,

236.

3. Yamamoto, S., Atomi, H., Ueda, M. & Tanaka, A. (1995) *Arch. Microbiol.* **163**, 104.
4. Jennings, G.T., Sechi, S., Stevenson, P.M., Tuckey, R.C., Parmelee, D. & McAlister-Henn, L. (1994) *J. Biol. Chem.* **269**, 23128.
5. Gould, S.J., Keller, G.-A. & Subramani, S. (1988) *J. Cell Biol.* **107**, 897.
6. Loftus, T.M., Hall, L.V., Anderson, S.L. & McAlister-Henn, L. (1994) *Biochemistry* **33**, 9661.
7. Haselbeck, R.J. & McAlister-Henn, L. (1993) *J. Biol. Chem.* **268**, 12116.
8. Zhao, W.-N. & McAlister-Henn, H. (1996) *Biochemistry* **35**, 7873.
9. Tajima, M., Nogi, Y. & Fukasawa, T. (1985) *Yeast* **1**, 67.
10. Kanai, T., Atomi, A., Umemura, K., Ueno, H., Teranishi, Y., Ueda, M. & Tanaka, A. (1996) *Appl. Microbiol. Biotechnol.* **44**, 759.
11. Kurihara, T., Ueda, M., Kanayama, N., Kondo, J., Teranishi, Y. & Tanaka, A. (1992) *Eur. J. Biochem.* **210**, 999.
12. Kaiser, C., Michaelis, S. & Mitchell, A. (1994) *Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press.*
13. Atomi, H., Ueda, M., Hikida, M., Hishida, T., Teranishi, Y. & Tanaka, A. (1990) *J. Biochem.* **107**, 262.
14. Benton, W.D. & Davis, R.W. (1977) *Science* **196**, 180.
15. Sanger, F., Nickelen, S. & Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463.

16. Brecker, D.M. & Guarente, L. (1991) *Methods Enzymol.* **194**, 182.

17. Ueda, M., Mozaffar, S., Atomi, H., Osumi, M. & Tanaka, A. (1989) *J. Ferment. Bioeng.* **68**, 411.

18. Nabeshima, S., Ishiyama, S., Tanaka, A. & Fukui, S. (1977) *Agric Biol. Chem.* **41**, 509

19. Lowry, O. H., Rosebrough, N.J., Farr, A. L. & Randall, R.J. (1951) *J. Biol. Chem.* **193**, 265.

20. Kimura, M. (1983) *The Neutral Theory of Molecular Evolution. Cambridge University Press, Cambridge.*

21. Saitou, N.M. & Nei, M. (1987) *Mol. Biol. Evol.* **4**, 406.

22. Felsenstein, J. (1985) *Evolution* **39**, 783.

23. Hikida, M., Atomi, H., Fukuda, Y., Aoki, A., Hishida, T., Teranishi, Y., Ueda, M. & Tanaka, A. (1991) *J. Biochem.* **110**, 909.

24. Haselbeck, R.J. & Mcalister-Henn, L. (1991) *J. Biol. Chem.* **266**, 2339.

25. Haselbeck, R.J., Colman, R.J. & Mcalister-Henn, L. (1992) *Biochemistry* **31**, 6219

26. Huh, T.-L., Ryu, J.-H., Huh, J.-W., Sung, H.-C., Oh, I. -U., Song, B.J. & Veech, R.L. (1993) *Biochem. J.* **292**, 705.

27. Udvardi, M.K., McDermott, T.R. & Kahn, M.L. (1993) *Plant Mol. Biol.* **21**, 739.

28. Scorrosh, B.S. & Dixon, R.A. (1992) *Plant Mol. Biol.* **20**, 801.

29. Miyazawa, S., Osumi, T., Hashimoto, T., Ohno, K., Miura, S. & Fujiki,

Y. (1989) *Mol. Cell. Biol.* **9**, 83.

30. Swinkels, B.W., Gould, S.J., Bodnar, A.G., Rachubinski, R.A. & Subramani, S. (1991) *EMBO J.* **10**, 3255.
31. Faber, K.N., Keizer-Gunnink, I., Pluim, D., Harder, W., AB, G. & Veenhuis, M. (1995) *FEBS Lett.* **357**, 115.
32. Kragler, F., Langeder, A., Raupachova, J., Binder, M. & Hartig, A. (1993) *J. Cell Biol.* **120**, 665.
33. Glover, J.M., Andrews, D.W., Subramani, S. & Rachubinski, R.A. (1991) *J. Biol. Chem.* **269**, 7558.
34. Small, G. M., Szabo, L. J. & Lazarow, P. B. (1988) *EMBO J.* 7, 1167.

Part II *Candida tropicalis* mitochondrial and peroxisomal carnitine acetyltransferases synthesized from one gene by alternative translation initiation

Chapter 1. Analyses of gene structure and translation products of *Candida tropicalis* mitochondrial and peroxisomal carnitine acetyltransferases

INTRODUCTION

Carnitine acyltransferases are a class of the enzymes that catalyze the reversible transfer of fatty acids between coenzyme A and carnitine:

$$\text{Acyl-CoA} + \text{Carnitine} \rightleftarrows \text{Acylcarnitine} + \text{CoASH}$$

In this class, the enzyme carnitine acetyltransferase (CAT) is distinct from carnitine palmitoyltransferase (1), which is specific for long chain fatty acids and from carnitine octanoyltransferase (2), which is most active toward medium chain fatty acids. CAT, catalyzing the transfer of short chain acyl groups, has been detected in most mammalian tissues (3, 4). In microorganisms, its existence was first demonstrated in the *n*-alkane-assimlating yeast, *Candida tropicalis* pK 233 (5). Unlike in mammalian cells (6, 7), in *Candida tropicalis* the fatty acid β-oxidation system exists only in peroxisomes (8). Therefore, peroxisomal and mitochondrial CATs constitute an "acetylcarnitine shuttle"

system between these organelles (9, 10). Acetyl-CoA, the final degradation product of the fatty acid β-oxidation in peroxisomes, is converted to acetylcarnitine by peroxisomal CAT, then acetylcarnitine being converted again to acetyl-CoA after being transported to mitochondria, in order to complete the operation of the tricarboxylic acid cycle in mitochondria. Recently, it has been reported that peroxisomal and mitochondrial CATs in *Saccharomyces cerevisiae* (11) are encoded by a single gene (12). Although the N-terminal amino acid sequences of both CATs have not been determined, alternative initiation sites for transcription has been suggested as responsible for the sorting of CATs to peroxisomes and mitochondria.

In this chapter, the author reports the nucleotide sequence of genomic DNA encoding both peroxisomal and mitochondrial CATs of *Candida tropicalis*. Moreover, from the results of the N-terminal amino acid sequences of purified CATs, the distinct translational initiation sites of the peroxisomal and mitochondrial enzymes are demonstrated.

MATERIALS AND METHODS

1. Strains and vectors

Escherichia coli DH-5α was used as a host cell for cloning and pUC19 as a plasmid for recombination. *Saccharomyses cerevisiae* MT8-1 (13) was used for the expression of the gene encoding CAT and pMW1 (14)

as a shuttle vector plasmid.

2. Preparation of probe

cDNA fragment from *Candida tropicalis* pK233 (ATCC 20336) encoding a part of CAT (15) was labeled as described in Part I, Chapter 1.

3. Screening of clones

The clones were screened from a λEMBL 3 genomic DNA library of *Candida tropicalis* cells, as described in Part I, Chapter 1 (16).

4. Restriction enzyme mapping and DNA sequencing

Restriction enzyme mapping and DNA sequencing were carried out as in Part I, Chapter 1 (17).

5. *CtCAT* gene expression plasmid pWCAT1

CtCAT1 (Fig. 1), isolated by screening, was used as the template for the polymerase chain reaction (PCR). *Bgl*II site at the 5' end and *Eco*RV site at the 3' end were introduced for the expression construct. The 3.1 kbp *Bgl*II-*Eco*RV fragment was amplified using the 5' terminal primer (5'-AAAGATCTCTACCCAATAAGG-3') and the 3' terminal primer (5'-GCGATATCCATTTGCGTTTCGTAC-3') oligonucleotides. This fragment was subcloned into the *Bgl*II-*Eco*RV site of pMW1,changing *Kpn*I site to *Bgl*II by *Bgl*II linker (Takara Shuzo, Kyoto, Japan). The resulting plasmid, pWCAT1, which is shown in Fig. 4, was transformed to *Saccharomyces cerevisiae* using the electroporation method (18).

6. Cultivation and preparation of cell-free extracts

Saccharomyces cerevisiae bearing the constructed plasmid was cultivated, as described in Part I, Chapter 2.

7. Cultivation and subcellular fractionation of *Candida tropicalis*

Candida tropicalis was cultivated and fractionated, as described in Part I, Chapter 2 (19-21).

Cell-free extracts from *Candida tropicalis* were prepared in the same manner as *Saccharomyces cerevisiae.*

8. Western blot analysis

An antibody against *Candida tropicalis* mitochondrial CAT (22) was used for detection of CAT. Sodium dodecyl sulfate-polyacrylamide gel (acrylamide 8.5 %) electrophoresis (SDS-PAGE) was carried out as in Part I, Chapter 1 (23).

9. N-terminal mino acid sequence analysis

Sequencing of N-terminal amino acid was performed as in Part I, Chapter 1.

10. Assay of enzymes and protein

Catalase and cytochrome *c* oxidase were assayed by the methods described by Fukui *et al.* (24). CAT activity was assayed at 30 °C by the 5,5'-dithiobis (2-nitrobenzoic acid) method or by following the increase in the absorbance of acetyl-CoA at 233 nm (25). Protein was quantified by the Lowry method (26).

RESULTS AND DISCUSSION

1. Identification of genomic DNA for carnitine acetyltransferase of *Candida tropicalis*

A λEMBL3 genomic DNA library of *Candida tropicalis* was screened for the gene encoding carnitine acetyltransferase (CAT) with the cDNA probe (about 700 bp) reported by Ueda *et al.* (15). Of the isolated clones, the clone having the longest insertion was named *CtCAT1*. The size was about 12 kbp, a part of which (about 4.4 kbp) was shown in Fig. 1. The region including a *Bam*HI-*Bam*HI fragment (about 900 bp) from *CtCAT1* (Fig. 1), which hybridized with the cDNA probe, was sequenced and an open reading frame composed of 1,881 bp, corresponding to 627 amino acid residues, was found (Fig. 2). The predicted molecular mass was 70,760 Da. This molecular mass corresponded well to that of the larger precursor of CAT synthesized *in vitro* (27). In the 5'-flanking region, there were two TATA boxes at positions -91 and -168. In the 3'-flanking region, there was a poly(A)-addition signal candidate located at position 1955.

2. Comparison of amino acid sequences for *Candida tropicalis* CAT and CATs from other sources

The amino acid sequences for *Candida tropicalis* CAT (CtCAT) and CATs from other sources (28-31) were compared (Fig. 3). Computer alignment revealed that *Candida tropicalis* enzyme possessed 46.3 % identity with

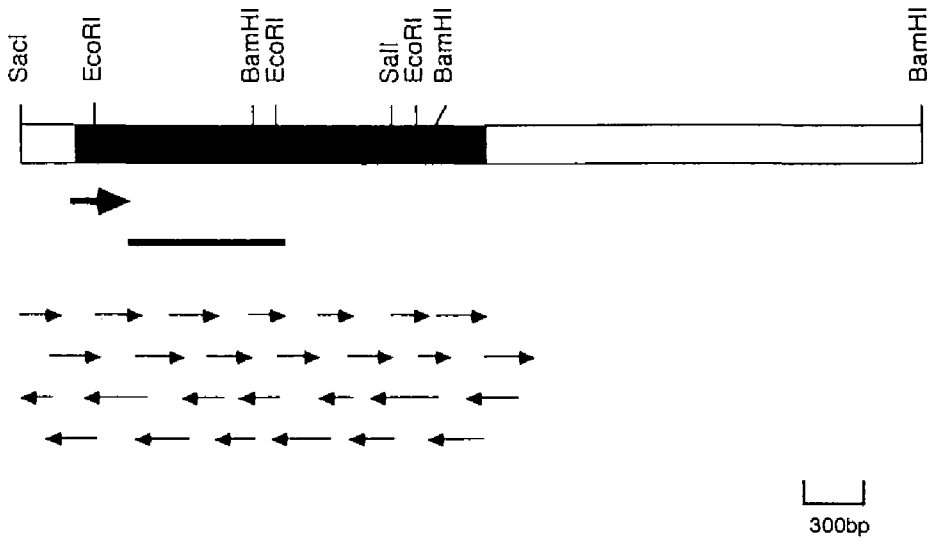


Fig. 1. Restriction map and sequencing strategy of the cloned *Sac*I-*Bam*HI genomic DNA fragment, a part of *CtCAT1*.

The thick arrow indicates the direction of transcription and the thin arrows indicate the direction and extent of sequence determinations. The thick bar corresponds to the cDNA portion. The closed box indicates the coding region.

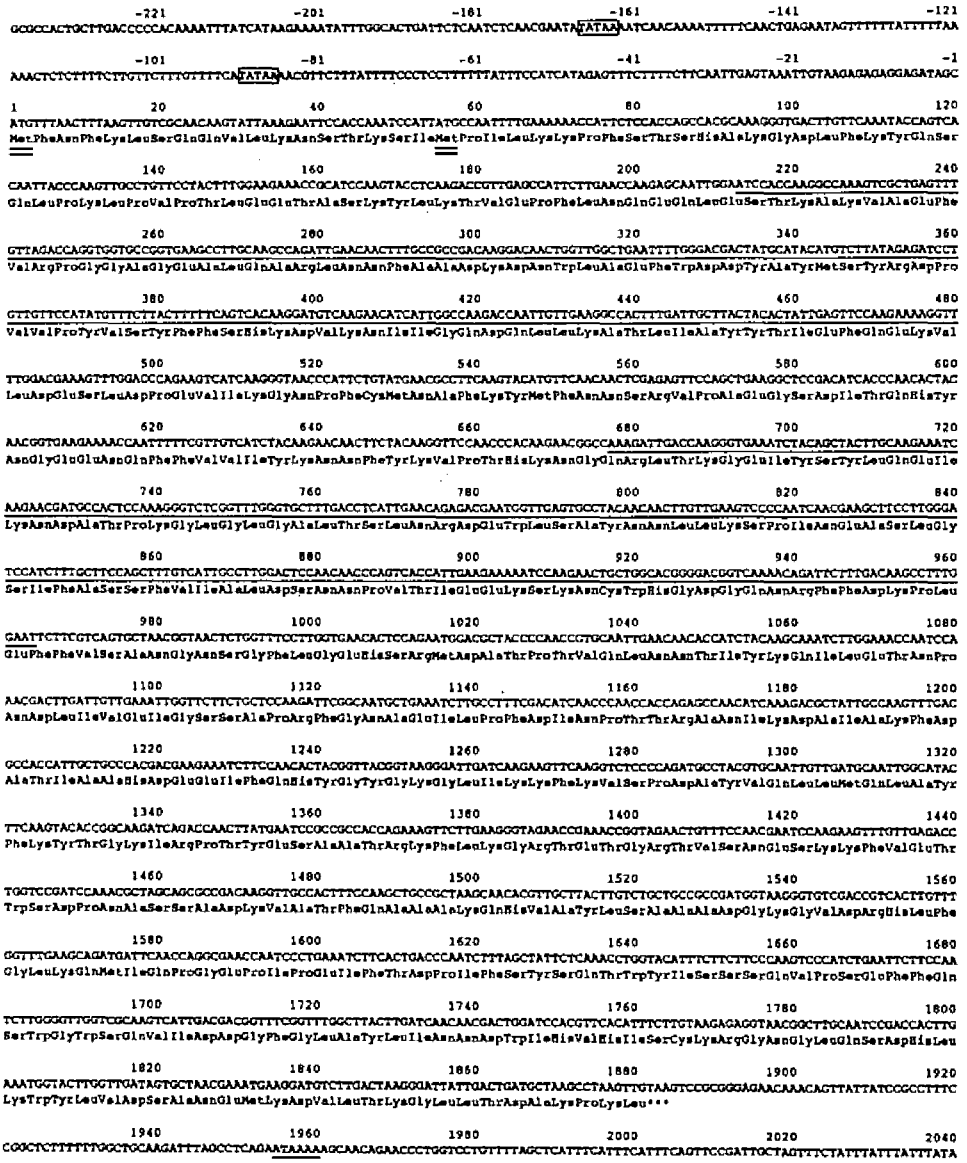


Fig. 2. Nucleotide sequence of the gene for CtCAT and its flanking regions. The amino acid sequence was deduced from the nucleotide sequence. Position 1 corresponds to the first nucleotide of the ATG initiation codon. TATA box sequences are boxed. In the coding region, two methionines are indicated in double underline. The underlined nucleotide sequences were identical to those of the isolated cDNA (see Fig. 1) analyzed partially from both ends. In the 3'-flanking region, a candidate of the poly(A) addition signal is underlined.

Fig. 3. Alignment of the amino acid sequences of CATs from *Candida tropicalis* (CtCAT), *Saccharomyces cerevisiae* (ScCAT1, ScCAT2), human (HCAT), and pigeon (PCAT).

All sequences are shown by the one-letter amino acid notation. Identical residues among CtCAT and others are indicated with '+'. Gaps have been inserted to achieve the maximum homology. The amino acids of each enzyme are numbered at their sides. The LPXLPXPXL motif (1) (33) and four more highly conserved regions (2-5) reported in (29) are underlined.

Saccharomyces cerevisiae mitochondrial matrix CAT (ScCAT1), and 30.8 % and 31.1 % with human CAT (HCAT) and pigeon CAT (PCAT), respectively, though CtCAT and *Saccharomyces cerevisiae* mitochondrial outer CAT (ScCAT2) displayed low identity (20.6 %). The LPXLPXPXL motif (32) (Fig. 3, underline(1)) and four typical motifs (28) (Fig. 3, underlines(2)-(5)) more highly conserved among choline acetyltransferases (pig and *Drosophila*) (33, 34) and other carnitine acyltransferases (rat carnitine octanoyltransferase (2) and rat and human carnitine palmitoyltransferases (1, 32)) were found in CtCAT. These conserved regions may be essential for the enzyme reaction, for instance, the binding sites for carnitine (choline), acylcarnitine, acyl-CoA or CoA.

3. Expression of *CtCAT* in *Saccharomyces cerevisiae*

Ueda *et al.* (22, 27) mentioned that peroxisomal and mitochondrial CATs consisted of two subunits, respectively. However, an inconsistent molar ratio of the smaller subunit compared with the larger subunit, suggested the

possibility that the smaller subunit might be a degradation product of the larger subunit. The author expressed the *CtCAT* gene in *Saccharomyces cerevisiae* MT8-1 under the control of its own promoter (Fig. 4). As shown in Table 1, the specific CAT activity of oleic acid-grown *Saccharomyces cerevisiae* bearing pWCAT1 (Fig. 4) was 12.5 times higher than that of the cells containing pMW1, indicating that the CtCAT 5'-upstream region was functional in *Saccharomyces cerevisiae* and that the product(s) of this gene alone was sufficient for CAT activity. On Western blot analysis (Fig. 5), no cross-reaction of the antibody against CtCAT with the *Saccharomyces cerevisiae* enzyme (Fig. 5, lane 3) was observed. Two faint 57 kDa and 52 kDa bands (arrowheads) were observed in lane 2, which corresponded to the sizes of the smaller subunits reported by Ueda *et al.* (22). These bands can also be detected in the cell-free extracts of *Candida tropicalis* (lane 1). Both samples displayed very low levels of the 57 kDa proteins, and considering their inconsistent ratio with the larger subunit, it can be concluded that the 57 kDa and 52 kDa polypeptides were degradation products of the larger subunits. Although the appearance of a single band corresponding to the larger subunit had been reported (22), under different electrophoresis conditions, this band was separated into two (about 64 kDa, arrows in Fig. 5) with the cell-free extracts of *Candida tropicalis* (lane 1) and also with the cell-free extracts of *Saccharomyces cerevisiae* harboring pWCAT1 (lane 2), indicating that both proteins are products of the *CtCAT* gene.

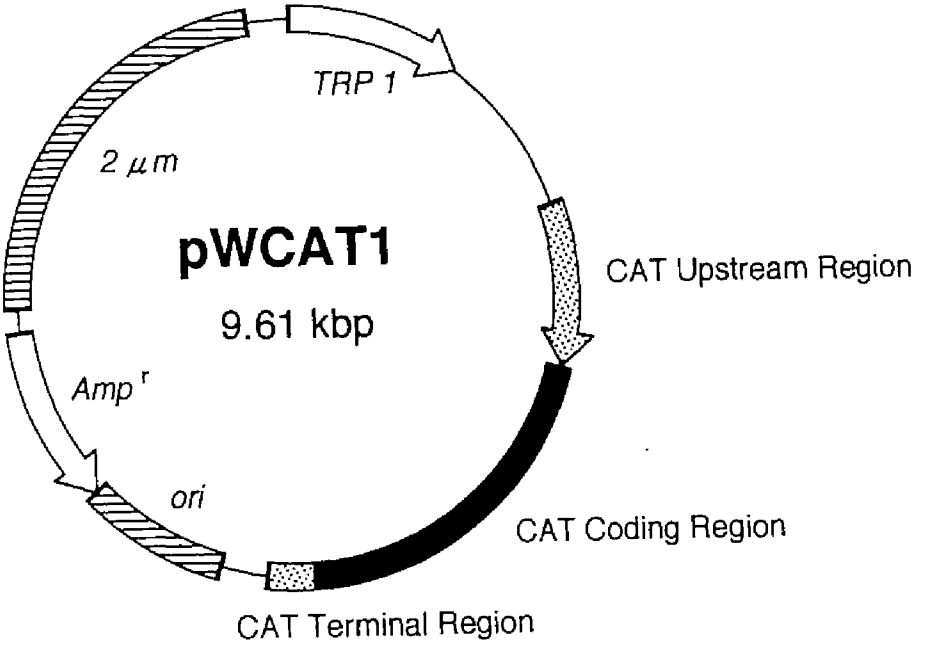


Fig. 4. Construction of the plasmid pWCAT1.

Table 1 Activity of CAT in Cell-free Extracts of *Saccharomyces cerevisiae* Harvoring pWCAT1.

Plasmid	Specific Activity ($nmol \cdot min^{-1} \cdot mg^{-1}$)
pWCAT1	65.0
pMW1	5.22

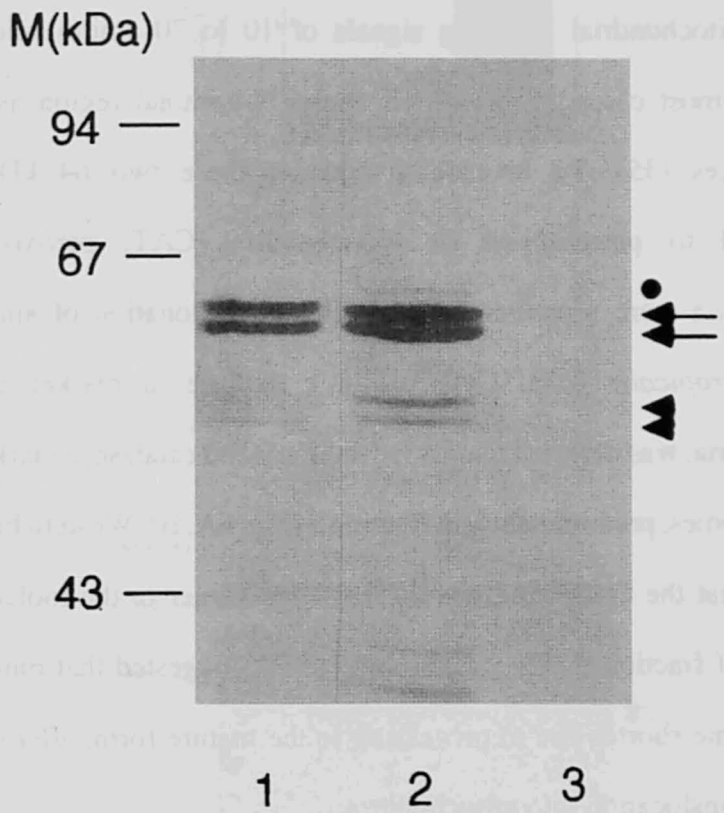


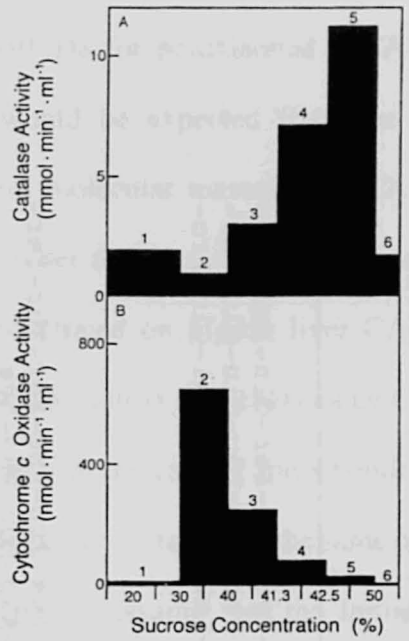
Fig. 5. Western blot analysis of cell-free extract prepared from *Saccharomyces cerevisiae* harboring pWCAT1 (lane 2) or control plasmid pMW1 (lane 3) and that from *Candida tropicalis* (lane 1).

Protein loaded, 15 μg each. The arrows and arrowheads indicate, so called, the 64 kDa and 57 kDa bands (22), respectively. A closed circle represents the band of the precursor form (see text). Molecular mass (M) of marker proteins: phosphoryrase b (94 kDa), albumin (67 kDa), and ovalbumin (43 kDa).

4. Peroxisomal and mitochondrial CATs

It is well known that the precursors of many mitochondrial proteins contain mitochondrial targeting signals of 10 to 70 amino acid residues, which, in most cases, are localized at the N-terminal region as cleavable presequences (35). To investigate whether these two 64 kDa proteins correspond to peroxisomal or mitochondrial CAT, peroxisomes and mitochondria were separated by subcellular fractionation of alkane-grown *Candida tropicalis* cells. Cytochrome *c* oxidase, a marker enzyme of mitochondria, was detected mainly in fraction 2 and catalase, a marker enzyme of peroxisomes, predominantly in fraction 5 (Fig. 6A, B). Western blot analysis revealed that the CAT band of fraction 5 was larger in the molecular mass than that of fraction 2 (Fig. 6C). These results suggested that mitochondrial CAT became shorter due to processing to the mature form, after or together with the translocation into mitochondria.

The author next analyzed the N-terminal amino acid sequences of peroxisomal and mitochondrial CATs from *Candida tropicalis*. After the peroxisomal and mitochondrial enzymes were separately purified by using DEAE-Sephacel columns (25), the amino acid sequences of the N-terminal region were analyzed by the Edman degradation procedure. As shown in Table 2, the N-terminal sequences of peroxisomal and mitochondrial CtCATs were "Pro-Ile-Leu-Lys-Lys-Pro-Phe-Ser-Thr-Ser-" and "Asp-Leu-Phe-Lys-Tyr-Gln-Ser-Gln-Leu-Pro-", respectively. If no other processing occurred,



C

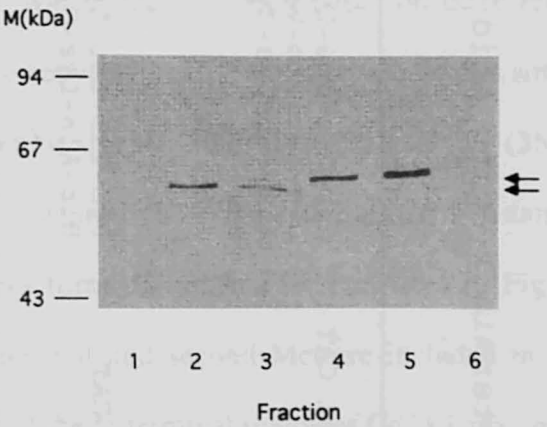


Fig. 6. Separation of peroxisomal and mitochondrial CATs by subcellular fractionation from the P_2 fraction of *n*-alkane-grown *Candida tropicalis*.

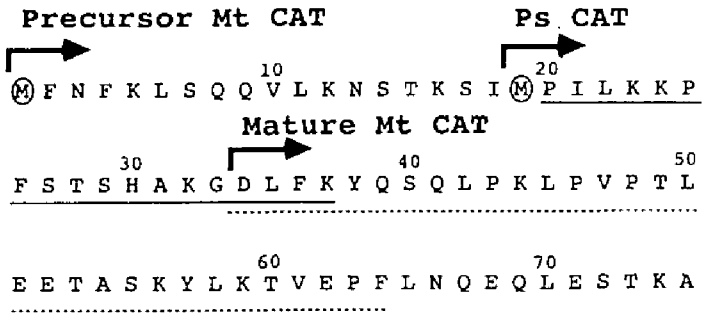
A, B The volume of each fraction was as follows: 1, 3.75 ml; 2-5, 2.5 ml each; 6, 1.25 ml. Catalase (A) was used as the peroxisomal marker enzyme and cytochrome *c* oxidase (B) as the mitochondrial marker enzyme. **C** Western blot analysis of the fractions obtained in this fractionation. Protein loaded, 2 μl each. The arrows represent the CAT subunits (see text).

Table 2 N-terminal Amino Acid Sequences of CtCATs.

Mitochondrial CAT	Asp-Leu-Phe-Lys-Tyr-Gln-Ser-Gln-Leu-Pro-Lys-Leu-Pro-Val-Pro-Thr-Leu-Glu-Glu-Thr-Ala-Ser-Lys-Tyr-Leu-Lys-Thr-Val-Glu-Pro-Phe
Peroxisomal CAT	Pro-Ile-Leu-Lys-Lys-Pro-Phe-Ser-Thr-Ser-His-Ala-Lys-Gly-Asp-Leu-Phe-Lys

molecular sizes of 68,191 Da for peroxisomal CtCAT and 66,446 Da for mitochondrial CtCAT would be expected from the deduced amino acid sequence. These apparent molecular masses of CtCAT measured by SDS-PAGE (64 kDa) were smaller than those predicted from the sequences. This tendency was also demonstrated on pigeon liver CAT (31). These results revealed that initiation of translation for peroxisomal CAT was at the second Met (residue 19) (Fig. 7A). In the case of mitochondrial CAT, on the other hand, there was also a possibility of starting at the same position as peroxisomal CAT. But it is reasonable to assume that the initiation of translation of mitochondrial CAT was at the first Met, based on three reasons. First, the amino acid sequence between the first and second Met was similar to proposed mitochondrial targeting signals. Helical wheel analysis (36) of this region revealed that basic residues (K) form one side of a putative helix, while hydrophobic residues form the other side, as shown in Fig. 7B, where the residues between the first and second Met are included in the inner circle. This amphiphilicity of the N-terminal region of CtCAT may play an important role in the transport of the precursor into mitochondria. Second, in Fig. 5, lane 2, a protein which migrated slower than the 68 kDa protein could be detected. This protein may be the precursor form of mitochondrial CAT, appeared due to the overproduction of the protein. Third, as reported recently, peroxisomal and mitochondrial CATs both encoded by the same gene were present in oleate-grown *Saccharomyces cerevisiae* (12). Two mRNAs are

A



B

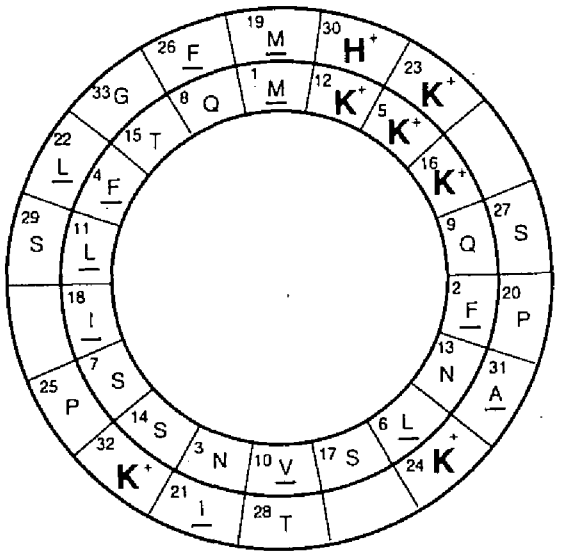


Fig. 7. The N-terminal region of CtCATs.

All amino acid residues are shown by the one-letter amino acid notation. The results of the amino acid sequence analysis of peroxisomal (solid line) and mitochondrial CAT (dotted line) are indicated, respectively. **A** The methionine residues are circled. **B** Amino acids of the N-terminal region are plotted on a helical wheel. Helical wheel analysis was performed according to the method of Schiffer and Edmundson (36). Basic amino acids are attached '+' and hydrophobic residues are underlined.

transcribed from different initiation sites, one from the upstream of the first Met, another between the first and second Met. It was supposed that the longer mRNA encodes the precursor for mitochondrial CAT which contains the N-terminal extention peptide and that the shorter mRNA lacks the N-terminal extension peptide, its product being translocated into peroxisomes. Though the author has not determined at present the transcriptional initiation sites for peroxisomal and mitochondrial CtCATs, the results obtained from amino acid sequence analysis have proved that the peroxisomal CAT did indeed start from the second Met. Here it should be noted that the C-terminal sequence of CtCAT was "-Pro-Lys-Leu", an analogue of "-Ser-Lys-Leu" which are proposed to act as a transport signal of proteins to peroxisomes (37, 38). Of several *Candida tropicalis* peroxisomal proteins whose amino acid sequences have already been known, PXP18 also has the same C-terminal sequence (39). However, it remains to be solved whether or not this C-terminal sequence of CtCAT actually serves as a targeting signal to peroxisomes. In the case of *Saccharomyces cerevisiae* CAT whose C-terminal sequence was "-Ala-Lys-Leu", the presence of an internal peroxisomal targeting signal was supposed, in addition to this C-terminal sequence (12).

The mechanisms of localization of proteins into various organelles have been studied extensively and several signal sequences in the protein molecules have been identified. CtCAT would be valuable for such studies from the viewpoint that CtCAT localizes in both peroxisomes and

mitochondria. Rat serine:pyruvate aminotransferase (SPT) is also the similar example that two proteins are encoded by one gene and are targeted to peroxisomes and mitochondria, respectively (40, 41). The signal sequences of these proteins will contribute to analyze the sorting mechanism among different organelles.

SUMMARY

A genomic DNA clone encoding carnitine acetyltransferases (CAT; EC 2.3.1.7) (*CTCAT1*), localized in two subcellular organelles, peroxisomes and mitochondria of an *n*-alkane-assimilating yeast, *Candida tropicalis*, was isolated from the yeast λEMBL library using a CAT cDNA probe. Nucleotide sequence analysis disclosed that the open reading frame was 1,881 bp, corresponding to 627 amino acids with a molecular mass of 70,760 Da. Comparison of the predicted amino acid sequence of *Candida tropicalis* CAT with that of *Saccharomyces cerevisiae* mitochondrial matrix CAT revealed 46.3 % identity. It was noticeable that *Candida tropicalis* CAT had amino acid sequences similar to both proposed mitochondrial and peroxisomal targeting signals. When this *CtCAT* gene was expressed in *Saccharomyces cerevisiae* using its own 5'-upstream region, a 12-fold increase in CAT activity was observed. Western blot analysis revealed the presence of two major proteins whose sizes corresponded to the peroxisomal and mitochondrial

CAT proteins detected in *Candida tropicalis* . This suggested that peroxisomal and mitochondrial CATs were encoded by one gene. Furthermore, the author purified CATs from peroxisomes and mitochondria of *Candida tropicalis*, and analyzed the N-terminal amino acid sequences of each CAT. The N-terminal sequence of the mitochondrial CAT suggested that an N-terminal signal sequence had been cleaved during translocation into mitochondria. Concerning peroxisomal CAT, the evidence obtained indicated that the translation of peroxisomal CAT was initiated at the second methionine of the open reading frame.

REFERENCES

1. Woeltje, K.F., Esser, V., Weis, B.C., Sen, A., Cox, W.F., McPhaul, M.J., Slaughter, C.A., Foster, D.W. & McGarry, J.D. (1990) *J. Biol. Chem.* **265**, 10720.
2. Chatterjee, B., Song, C.S., Kim, J.M. & Roy, A.K. (1988) *Biochemistry* **27**, 9000.
3. Fritz, I.B., Schultz, S.K. & Srere, P.A. (1963) *J. Biol. Chem.* **238**, 2509.
4. Mittal, B. & Kurup, C.K.R. (1980) *Biochim. Biophys. Acta* **619**, 90.
5. Kawamoto, S., Ueda, M., Nozali, C., Yamamura, M., Tanaka, A. & Fukui, S. (1978) *FEBS Lett.* **96**, 37.
6. Lazarow, P.B. & de Duve, C. (1976) *Proc. Natl. Acad. Sci. USA* **73**,

2043.

7. Masters, C. & Crane, D. (1984) *Trends Biochem. Sci.* **9**, 314-317.
8. Kawamoto, S., Nozaki, A., Tanaka, A. & Fukui, S. (1978) *Eur. J. Biochem.* **83**, 609.
9. Tanaka, A. & Ueda, M. (1993) *Mycol. Res.* **97**, 1025.
10. Fukui, S. & Tanaka, A. (1979) *Trends Biochem. Sci.* **4**, 246.
11. Atomi, H., Ueda, M., Suzuki, J., Kamada, Y. & Tanaka, A. (1993) *FEMS Microbiol. Lett.* **112**, 31.
12. Elgersma, Y., Van Roermund, C.W.T., Wamders, R.J., A. & Tabak, H.F. (1995) *EMBO J.* **14**, 3472.
13. Tajima, M., Nogi, Y. & Fukasawa, T. (1985) *Yeast* **1**, 67.
14. Kanai, T., Atomi, A., Umemura, K., Ueno, H., Teranishi, Y., Ueda, M. & Tanaka, A. (1996) *Appl. Microbiol. Biotechnol.* **44**, 759.
15. Ueda, M., Okada, H., Hishida, T., Teranishi, Y. & Tanaka, A. (1987) *FEBS Lett.* **220**, 31.
16. Benton, W.D. & Davis, R.W. (1977) *Science* **196**, 180.
17. Sanger, F., Nickelen, S. & Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463.
18. Brecker, D.M. & Guarente, L., (1991) *Methods Enzymol.* **194**, 182.
19. Tanaka, A., Yamamura, M., Kawamoto, S. & Fukui, S. (1977) *Appl. Environ. Microbiol.* **34**, 342.
20. Kurihara, T., Ueda, M. & Tanaka, A. (1988) *FEBS Lett.* **229**, 215.

21. Ueda, M., Yamanoi, K., Morikawa, T., Okada, H. & Tanaka, A. (1985) *Agric. Biol. Chem.* **49**, 1821.

22. Ueda, M., Tanaka, A. & Fukui, S. (1984) *Eur. J. Biochem.* **138**, 445.

23. Ueda, M., Mozaffar, S., Atomi, H., Osumi, M. & Tanaka, A. (1989) *J. Ferment. Bioeng.* **68**, 411.

24. Fukui, S., Kawamoto, S., Yasuhara, S., Tanaka, A., Osumi, M. & Imaizumi, F. (1975) *Eur. J. Biochem.* **59**, 561.

25. Ueda, M., Tanaka, A. & Fukui, S. (1982) *Eur. J. Biochem.* **124**, 205.

26. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.

27. Ueda, M., Tanaka, A., Horikawa, S., Numa, S. & Fukui, S. (1984) *Eur. J. Biochem.* **138**, 451.

28. Kispal, G., Sumegi, B., Dietmeier, K., Bock, I., Gajdos, G., Tomcsanyi, T. & Sandor, A. (1993) *J. Biol. Chem.* **268**, 1824.

29. Schmalix, W. & Bandlow, W. (1993) *J. Biol. Chem.* **268**, 27428.

30. Corti, O., Finocchiaro, G., Rossi, E., Zuffardi, O. & Didonato, S. (1994) *GENOMICS* **23**, 94.

31. Johnson, T.M., Kocher, H.P., Anderson, R.C. & Nemecek, G.M. (1995) *Biochem. J.* **305**, 439.

32. Finocchiaro, G., Taroni, F., Rocchi, M., Martin, A.L., Colombo, I., Tarelli, G.T. & DiDonato, S. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 661.

33. Berrard, S., Brice, A., Lottspeich, F., Braun, A., Barde, Y. & Mallet, J.

(1987) *Proc. Natl. Acad. Sci. USA* **84**, 9280.

34. Brice, A., Berrard, S., Raynaud, B., Ansieau, S., Coppola, T., Weber, M.J. & Mallet, J. (1989) *J. Neurosci. Res.* **23**, 266.

35. Hartl, F.-U., Pfanner, N., Nicholson, D.W. & Neupert, W. (1989) *Biochim. Biophys. Acta* **988**, 1.

36. Schiffer, M. & Edmundson, A.B. (1967) *Biophys. J.* 7, 121.

37. Gould, S.J., Keller, G.-A. & Subramani, S. (1988) *J. Cell Biol.* **107**, 897.

38. Miyazawa, S., Osumi, T., Hashimoto, T., Ohno, K., Miura, S. & Fujiki, Y. (1989) *Mol. Cell. Biol.* **9**, 83.

39. Tan, H., Okazaki, K., Kubota, I., Kamiryo, T. & Utiyama, H. (1990) *Eur. J. Biochem.* **190**, 107.

40. Oda, T., Miyajima, H., Suzuki, Y. & Ichiyama, A. (1987) *Eur. J. Biochem.* **168**, 537.

41. Oda, T., Funai, T. & Ichiyama, A. (1990) *J. Biol. Chem.* **265**, 7513.

Chapter 2. Individual expression of *Candida tropicalis* mitochondrial and peroxisomal carnitine acetyltransferase-encoding genes and subcellular localization of the products in *Saccharomyces cerevisiae*

INTRODUCTION

Carnitine acetyltransferase (CAT), which catalyzes the reversible transfer of acetyl group between coenzyme A and carnitine, is localized in two subcellular organelles, peroxisomes and mitochondria, in an *n*-alkane-assimilating yeast, *Candida tropicalis* (1). When *Candida tropicalis* is grown on *n*-alkanes as the sole carbon source, acetyl-CoA is formed as the final degradation product of the β-oxidation system in peroxisomes. CATs localized in peroxisomes and mitochondria form an "acetylcarnitine shuttle", allowing the transfer of acetyl unit from peroxisomes to mitochondria (2, 3).

As described in the preceding chapter, the subunit of mitochondrial CAT of *Candida tropicalis* had molecular masse of 66 kDa, while that of the peroxisomal CAT was 68 kDa. Screening from a λEMBL genomic DNA library of the yeast led to the isolation of DNA fragments all containing the same open reading frame corresponding to a protein of 71 kDa, which showed homology with previously reported CAT genes from other sources. When the gene was introduced into the yeast *Saccharomyces cerevisiae* under the

control of its own promoter, an increase in CAT activity was demonstrated in the cells and proteins with sizes of 66 and 68 kDa could be detected by Western blot analysis using the antibody against *Candida tropicalis* CAT (CtCAT). This suggested that the two proteins localized in different organelles were the products of one single gene. Furthermore, CATs from peroxisomes and mitochondria of *Candida tropicalis* were purified to analyze their N-terminal amino acid sequences. The N-terminus of peroxisomal CAT was "Pro-Ile-Leu-Lys-" which directly followed the second Met in the open reading frame. That of mitochondrial CAT was "Asp-Leu-Phe-Lys-", further downstream in the open reading frame and not adjacent to Met. Based on these results, the author proposed that the translation of peroxisomal CAT was initiated at the second Met (residue no. 19) of the open reading frame, and that, in the case of mitochondrial CAT, the initiation of translation was at the first Met, because there was a proposed mitochondrial targeting sequence, an amphiphilic helix, between the first and second Met, where basic residues form one side, while hydrophobic residues form the other side as described in Part II, Chapter 1.

In the case of *Saccharomyces cerevisiae* CAT, a single gene encodes both peroxisomal and mitochondrial CATs and alternative initiation sites for transcription have been suggested as responsible for the sorting of CATs to peroxisomes and mitochondria. A polypeptide encoded by the longer transcripts, which contain the N-terminal extension peptide, is targeted to

mitochondria, whereas that encoded by the shorter mRNA, which lacks the N-terminal extension peptide, is targeted to peroxisomes (4). But there is no specific information about the C-terminal amino acid sequences of both CATs.

In this chapter, the author individually expressed *Candida tropicalis* peroxisomal and mitochondrial carnitine acetyltransferase-encoding genes in *Saccharomyces cerevisiae*, followed by Western blot analysis and immunocytochemical study of the subcellular distribution, and discussed the sorting mechanism of peroxisomal and mitochondrial CATs.

MATERIALS AND METHODS

1. Strain

Saccharomyces cerevisiae MT8-1 (5) was used to investigate the proliferation of peroxisomes in a medium containing oleic acid (6).

2. Construction of expression plasmids pWICM1 and pWICM2

As described in the preceding chapter, the author isolated the gene *CtCAT1* encoding CATs from the λEMBL library of *Candida tropicalis* using a CAT cDNA probe (7). This *CtCAT* was used as the template for polymerase chain reaction (PCR). *Sal*I site at the 5' end and *Nco*I site at the 3' end were introduced for the construction of expression plasmids. M1 fragment, including the first ATG corresponding to mitochondrial CAT, was amplified with the No. 1 primer (5'-TGTAAGAGAGATCTTCAACCATGTT

TAACT-3') and the No. 2 primer (5'-TTCTCCCATGGACTTACAACTTAG GCTTAG-3'). M2 fragment, starting from the second ATG, corresponding to peroxisomal CAT, was made using the No. 3 primer (5'-AATTAGATCTAATC AACCATGCCAATTTTG-3') and the No. 2 primer. The oligonucleotide primers were synthesized by a 381A DNA Synthesizer (Applied Biosystems, Foster City, CA, U.S.A.). These *Sal*I-*Nco*I fragments, M1 and M2, were subcloned into the *Sal*I-*Nco*I site of pWI3, harboring the ICL promoter gene (*UPR-ICL*) (8) (Fig. 1A, B). These plasmids, pWICM1 for the expression of mitochondrial CAT and pWICM2 for the expression of peroxisomal CAT, were transformed to *Saccharomyces cerevisiae* using the electroporation method as described in Part I, Chapter 1 (9).

3. Cultivation

Saccharomyces cerevisiae was cultivated as in Part I, Chapter 2. After precultivation in YPD medium, cells were transferred to YPO medium (1 % yeast extract, 1 % peptone and 0.5 % oleic acid by weight).

4. Preparation of cell-free extracts

Cell-free extracts were prepared, as descrived in Part I, Chapter 2.

5. Subcellular fractionation

The subcellular fractionation of *Saccharomyces cerevisiae* cells was carried out by the method as described by Atomi *et al.* (6) with some modification: 2.5 mM potassium phosphate buffer (pH 7.2) containing 0.65 M sorbitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 50 μg/ml

pepstatin A, 50 μg/ml leupeptin, 50 μg/ml antipain, and 50 μg/ml chymostatin (Peptide Institute, Inc., Osaka, Japan) was used for protoplast homogenization. After the homogenate was centrifuged at 3,000 x *g* for 10 min, the supernatant (S_1) obtained was subjected to centrifugation at 20,000 x *g* for 15 min to obtain P_2 fraction, pellet containing organelles and S_2 fraction, supernatant containing cytosol.

6. Western blot analysis

Western blot analysis using anti-CtCAT antibody was carried out as in Part I, Chapter 1 (10, 11).

7. Electronmicroscopy and immunoelectronmicroscopy

For electronmicroscopy, the cells were prefixed with 2.5 % glutaraldehyde, and post-fixed with $KMnO_4$ (12). For immunocytochemical labeling, yeast cells were fixed with a mixture of 0.5 % glutaraldehyde (Nisshin EM Co., Tokyo, Japan) and 3 % paraformaldehyde (TA AB Laboratories Equipment Ltd., Berkshire, England) in PBS buffer (0.1 M potassium phosphate buffer (pH 7.6) containing 0.8 % NaCl). A gold-particle-labeled goat anti-rabbit IgG was used as the second antibody (13). As pWICM1 transformant cells, the cells cultivated for 2 h were used, because poor morphology of mitochondria in the cells was observed when cultivated for the same period as the pWICM2 transformant cells (20 h).

8. Measurement of CAT activity and protein

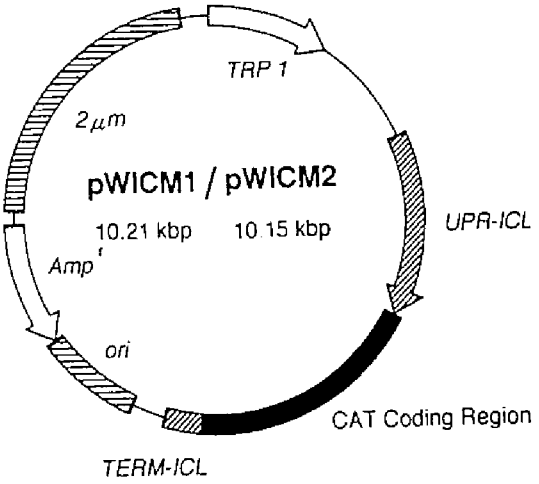
CAT activity and protein were assayed by the methods descrived in the preceding chapter (1).

RESULTS AND DISCUSSION

1. Expression of *CtCAT* using the upstream region of *Candida tropicalis* isocitrate lyase Gene (*UPR-ICL*) in *Saccharomyces cerevisiae*

Peroxisomal and mitochondrial CtCATs have been suggested to be translated from different initiation sites in one chromosomal gene, as described in the preceding chapter, based on the results of analyses on the *CtCAT* gene and the N-terminal amino acids of the purified enzymes. Therefore, the author constructed plasmids which permit individual expression from the first and second ATG codons of the *CtCAT* gene, and then observe the size and the localization of the protein products in *Saccharomyces cerevisiae*. The isocitrate lyase promoter from *Candida tropicalis* (*UPR-ICL*) was employed, because this promoter induces gene expression in *Saccharomyces cerevisiae* when the cells are grown on oleic acid (14, 15). *Saccharomyces cerevisiae* MT8-1 bearing pWICM1 starting from the first Met codon or pWICM2 starting from the second Met codon (Fig. 1A, B) was cultivated in the oleic acid medium to induce CAT synthesis and to proliferate peroxisomes. As shown in Table 1, the activity of the cells bearing pWICM1 was 10.8 times higher than that of the cells containing pMW1 as a control plasmid. Much higher

A



B

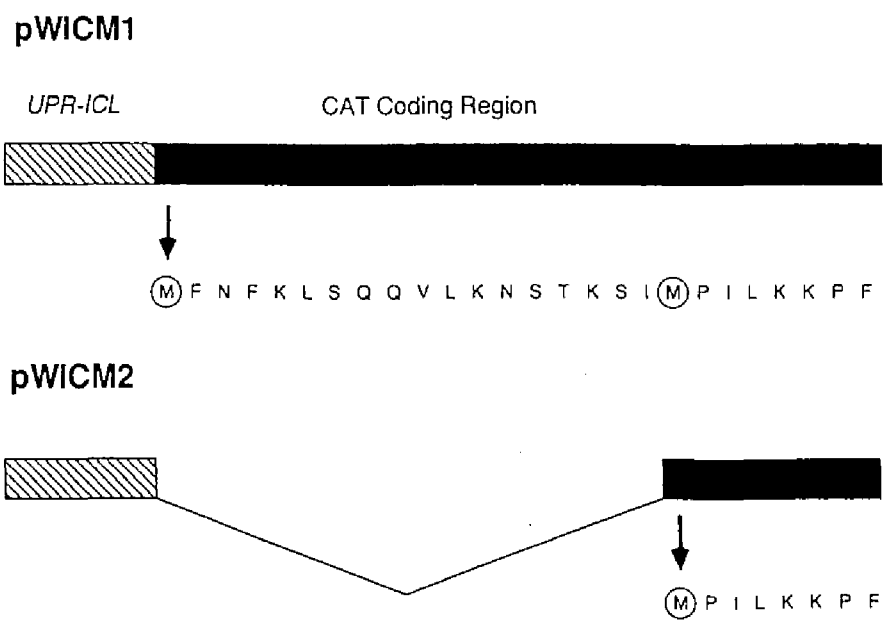


Fig. 1. Construction of plasmids pWICM1 and pWICM2 (A). The joint sites between *UPR-ICL* and *CtCAT* gene of pWICM1 and pWICM2 were shown by amino acid residues (B).

Table 1 Activity of CAT in Cell-free Extracts *Saccharomyces cerevisiae* Harboring Plasmids.

Plasmid	Specific Activity ($nmol \cdot min^{-1} \cdot mg^{-1}$)
pWICM1	56.3
pWICM2	351.0
pMW1	5.22

level induction of CAT was observed in the cells bearing pWICM2, initiating the translation at the second Met (residue no. 19), the amount of recombinant CAT being estimated to be as much as 31 % of the total extractable proteins in *Saccharomyces cerevisiae* from the densitometric measurement using a Shimadzu CS-9000 densitometer and the enzyme activity in this pWICM2 transformant being 67.2 times higher than that in the pMW1-transformed cells.

2. Detection of 71 kDa precursor of mitochondrial type CAT

The author suggested previously that the translation of mitochondrial CAT was initiated at the first Met, because the protein seemed to be processed after translocation into mitochondria and to have a putative mitochondrial targeting signal located between the first and second Met. If this was the case, a 71 kDa precursor would be produced in cytosol which would be processed to the 66 kDa protein found in mitochondria. By overexpression of the *CtCAT* gene in pWICM1 followed by Western blot analysis (Fig. 2, lane 2), a 71 kDa precursor protein of CAT was surely detected along with the 66 kDa mature CAT protein. This is a strong evidence that 71 kDa precursor synthesized, initiated at the first Met, was converted to the mature size (66 kDa) through processing and that the 66 kDa protein was formed by processing of the 71 kDa polypeptide, but was not a processed or degraded protein of the 68 kDa polypeptide (peroxisomal CAT) (lane 3). It is interesting that both peroxisomal and mitochondrial CAT proteins appeared when *CtCAT*

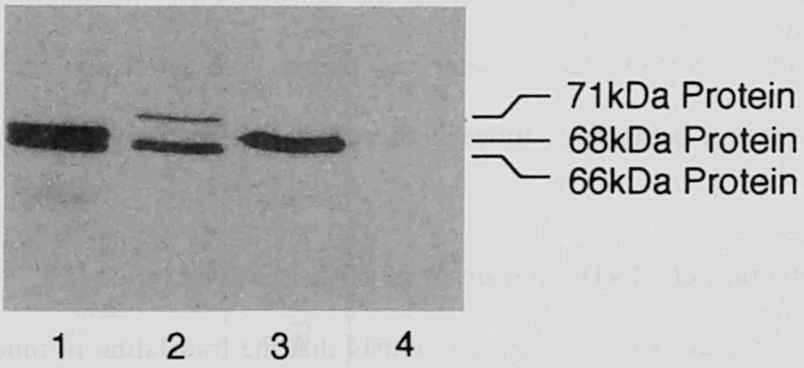


Fig. 2. Western blot analysis of cell-free extracts (1μg protein) prepared from *Saccharomyces cerevisiae* harboring pWICM1 (lane 2), pWICM2 (lane 3), and control plasmid pMW1 (lane 4), after 20 h cultivation in YPO medium. CtCAT was run on lane 1.

was expressed under the control of the CAT promoter as described in the preceding chapter, while the 68 kDa protein was not synthesized under the control of *UPR-ICL*. Therefore, the CAT original promoter seemed to be essential for the simultaneous expression of two types of CATs from one gene, *CtCAT*. Similar results were also demonstrated in the case of *Saccharomyces cerevisiae* CAT (4). In contrast, only a 68 kDa protein was observed in the cells bearing the plasmid pWICM2. There was no cross-reaction of the antibody against CAT of *S.cerevisiae* (lane 4). The amino acid sequences of the N-terminal regions of these recombinant CATs (66 kDa and 68 kDa proteins) produced in *Saccharomyces cerevisiae* analyzed by Edman degradation were identical with those of peroxisomal and mitochondrial CATs in *Candida tropicalis*, that is, "Pro-Ile-Leu-Lys-Lys-Pro-Phe-Ser-Thr-Ser-" and "Asp-Leu-Phe-Lys-Tyr-Gln-Ser-Gln-Leu-Pro-", respectively.

3. Immunocytochemical staining of *Candida tropicalis* CATs in transformed *Saccharomyces cerevisiae* cells

In order to investigate the localization of recombinant CtCATs in *Saccharomyces cerevisiae*, electronmicroscopical study was carried out (Fig. 3). In the pWICM2 transformant cells, the produced CAT protein was detected only in cytosol and peroxisomes, but not in mitochondria (Fig. 3C, D). Many positive signals observed in cytosol might be resulted from the overproduction of CAT by using of *UPR-ICL* as reported (16). The results indicated that the peroxisomal targeting signal of CtCAT seemed to be functional in

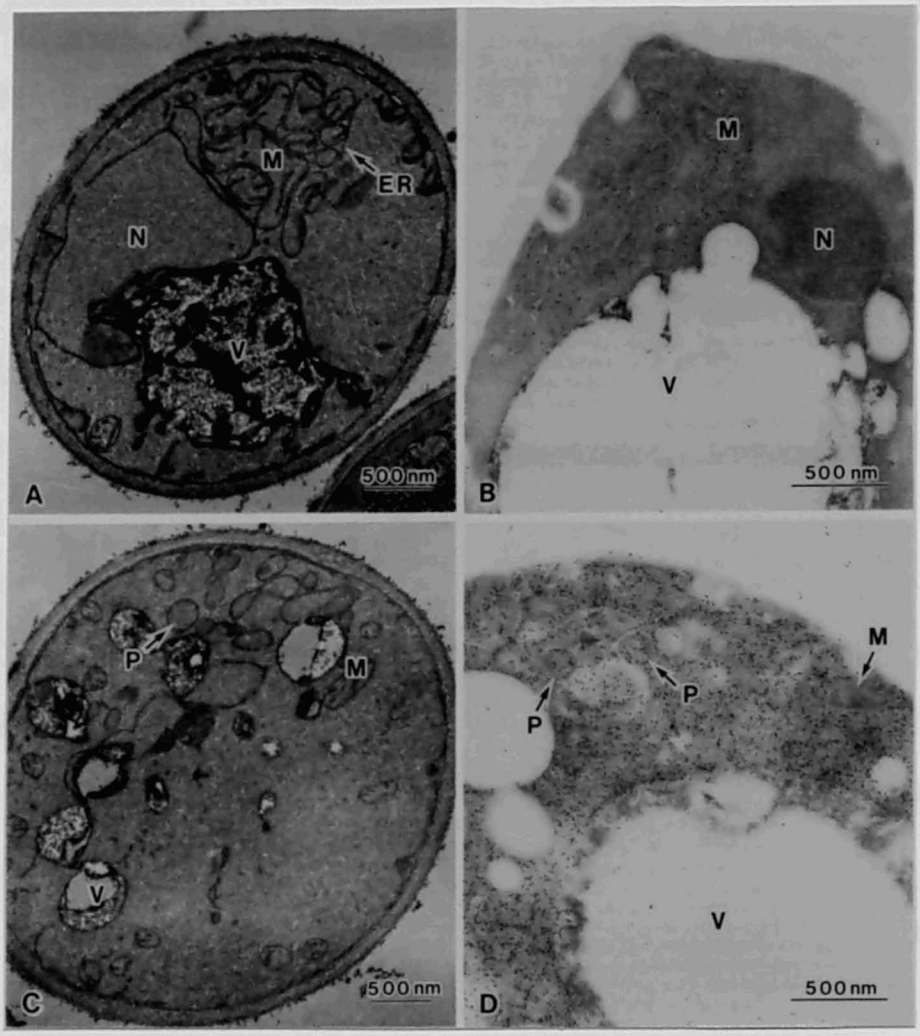


Fig. 3. Electronmicrographs of whole cells fixed with KMnO4 (A, C) and immunoelectronmicrographs (B, D) with anti-CtCAT antibody of *Saccharomyces cerevisiae* MT8-1 harboring pWICM1 (A, B) and pWICM2 (C, D), respectively. M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole; ER, endoplasmic reticulum.

Saccharomyces cerevisiae. In the case of pWICM1 transformant cells, CAT was localized mostly in mitochondria (Fig. 3A, B). These results strongly suggested that the 66kDa CAT protein was localized in mitochondria, after cleavage of a mitochondrial targeting signal of the 71 kDa precursor (Fig. 2, lane 2). This was confirmed by subcellular fraction of pWICM1 transformant cells (Fig. 4, lane 2). The 71 kDa band seen in Fig. 4, lane 2 seemed to be a precursor CAT before transport into mitochondrial matrix, which also supported by the signals observed in mitochondrial membranes in Fig. 3B. The 66 kDa CAT protein was actually detected only in the P_2 fraction containing organelles (mitochondria) and not in the S_2 fraction (cytosol).

In the case of rat serine:pyruvate aminotransferase (SPT), when a cDNA encoding the precursor of mitochondrial SPT was expressed in COS cells, SPT was present predominantly in mitochondria, but a small number of peroxisomes were also positively stained (17, 18), owing to leaky scanning by the 40s ribosomal subunit (19). However, the author could not detect leaky scanning in the cells bearing the plasmid pWICM1 so far as *UPR-ICL* was used as the promoter. This is also the same in *Saccharomyces cerevisiae* CAT that was found to be localized only in mitochondria, when the gene starting from the first ATG codon was expressed under the control of the *Saccharomyces cerevisiae* catalase A promoter (4).

Based on the results obtained, the author can conclude that peroxisomal and mitochondrial CATs arise by the different initiation of translation on one

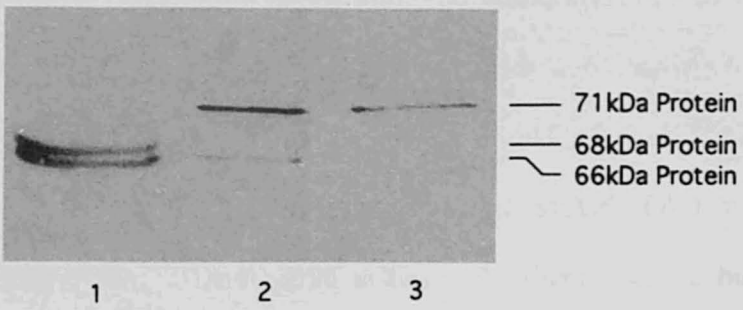


Fig. 4. Western blot analysis of P_2 fraction (3 μl) (lane 2) and S_2 fraction (13.6 μl) (lane 3) prepared from *Saccharomyces cerevisiae* harboring pWICM1, after 2 h cultivation in YPO medium. CtCAT was run on lane 1.

gene, *CtCAT*. When 71 kDa CAT, initiating translation at the first Met, was expressed, CAT immunoreactivity was observed in mitochondria. Moreover, the N-terminal mitochondrial signal must be removed from the precursor after the translocation into mitochondria. Only the 71 kDa protein gave rise to 66 kDa protein. On the other hand, CAT was found to be localized in peroxisomes and cytosol, but not in mitochondria and vacuoles, when 68 kDa CAT, initiating translation at the second Met (residue no. 19), was expressed.

In CtCAT, *Saccharomyces cerevisiae* CAT, and rat SPT, the mitochondrial proteins have a putative peroxisomal targeting signal in addition to the mitochondrial targeting signal. Since the 68 kDa CAT protein starting from the second Met localizes to peroxisomes, the targeting signal must be present in this protein. The mitochondrial targeting signal located at the N-terminus of the protein seems not only to direct protein transportation to mitochondria, but also to abolish the effect of the peroxisomal targeting signal which still should be present in the protein molecule. It is interesting to clarify the mechanism how the mitochondrial targeting signal completely overrides the peroxisomal targeting signal.

SUMMARY

In an *n*-alkane-assimilating yeast, *Candida tropicalis*, carnitine

acetyltransferase (CAT; EC 2.3.1.7) was localized in both peroxisomes and mitochondria. Both CATs were encoded by one gene, *CtCAT*, although the initiation sites of translation were suggested to be different. In the present chapter, the genes corresponding to *Candida tropicalis* peroxisomal and mitochondrial CATs, which were truncated from the *CtCAT* gene, were individually expressed in *Saccharomyces cerevisiae*, using the *Candida tropicalis* isocitrate lyase promoter (*UPR-ICL*), which is inducible by oleic acid in harmony with proliferation of peroxisomes in *Saccharomyces cerevisiae* (15). The 71 kDa precursor of mitochondrial CAT, initiating at the first Met, was found to be processed to the mature size (66 kDa) in *Saccharomyces cerevisiae* and immunoelectronmicroscopical observation revealed that this enzyme was localized in mitochondria. On the other hand, 68 kDa CAT, initiating at the second Met (residue no. 19), had no cleavable signal and was translocated into peroxisomes and cytosol, but not into mitochondria. The N-terminal amino acid sequences of individually expressed CATs were identical to those of CATs isolated from alkane-grown *Candida tropicalis* cells, respectively. These results demonstrated that only the 71 kDa protein yielded the 66 kDa protein and that peroxisomal and mitochondrial CATs arose from the difference in the initiation sites of translation.

REFERENCES

1. Kawamoto, S., Ueda, M., Nozaki, C., Yamamura, M., Tanaka, A. & Fukui, S. (1978) *FEBS Lett.* **96**, 37.
2. Ueda, M., Tanaka, A. & Fukui, S. (1982) *Eur. J. Biochem.* **124**, 205.
3. Fukui, S. & Tanaka, A. (1979) *Trends Biochem. Sci.* **4**, 246.
4. Elgersma, Y., Van Roermund, C.W.T., Wamders, R.J.A. & Tabak, H.F. (1995) *EMBO J.* **14**, 3472.
5. Tajima, M., Nogi, Y. & Fukasawa, T. (1985) *Yeast* **1**, 67.
6. Atomi, H., Ueda, M., Suzuki, J., Kamada, Y. & Tanaka, A. (1993) *FEMS Microbiol. Lett.* **112**, 31.
7. Ueda, M., Okada, H., Hishida, T., Teranishi, Y. & Tanaka, A. (1987) *FEBS Lett.* **220**, 31.
8. Kanai, T., Atomi, H., Umemura, K., Ueno, H., Teranishi, Y., Ueda, M. & Tanaka, A. (1996) *Appl. Microbiol. Biotechnol.* **44**, 759.
9. Brecker, D. M. & Guarente, L. (1991) *Methods Enzymol.* **194**, 182.
10. Ueda, M., Tanaka, A. & Fukui, S. (1984) *Eur. J. Biochem.* **138**, 445.
11. Ueda, M., Mozaffar, S., Atomi, H., Osumi, M. & Tanaka, A. (1989) *J. Ferment. Bioeng.* **68**, 411.
12. Numata, K., Ueki, T., Naito, N., Yamada, N., Kamasawa, N., Oki, T. & Osumi, M. (1993) *J. Electron Microsc.* **42**, 147.
13. Kamasawa, N. Ohtsuka, I., Kamada, Y., Ueda, M., Tanaka, A. & Osumi,

M. (1996) *Cell Struct. Funct.* **21**, 117.

14. Atomi, H., Umemura, K., Higashijima, T., Kanai, T., Yotsumoto, Y., Teranishi, Y., Ueda, M. & Tanaka, A. (1995) *Arch. Microbiol.* **163**, 322.
15. Umemura, K., Atomi, H., Kanai, T., Teranishi, Y., Ueda, M. & Tanaka, A. (1995) *Appl. Microbiol. Biotechnol.* **43**, 489.
16. Kamada, Y., Ueda, M., Atomi, H., Oda, K., Kurihara, T., Naito, N., Ukita, R., Kamasawa, N., Osumi, M. & Tanaka, A. (1992) *J. Ferment. Bioeng.* **74**, 368.
17. Yokota, S., Funai, T. & Ichiyama, A. (1991) *Biomed. Res.* **12**, 53.
18. Funai, T. & Ichiyama, A. (1995) *J. Biochem.* **117**, 1008.
19. Kozak, M. (1989) *J. Cell Biol.* **108**, 229.

Chapter 3. Alternative translation initiation from the transcripts of a single gene encoding mitochondrial and peroxisomal carnitine acetyltransferases of *Candida tropicalis*

INTRODUCTION

As described in the previous chapters of this Part, both mitochondrial and peroxisomal carnitine acetyltransferases (CATs) in an *n*-alkane-assimilating yeast, *Candida tropicalis* are encoded by one gene, *CtCAT*. These mitochondrial and peroxisomal isozymes differ from each other in their N-terminal regions.

There are many examples of other yeast genes that code for proteins found in more than one subcellular compartment (1). These mechanisms include alternative forms of transcription initiation and translation initiation. The examples of alternative transcription initiation were reported in yeast *Saccharomyces cerevisiae*, in the cases of *SUC2* (2), *HTS1* (3), *FUM1* (4), *VAS1* (5), *TRM1* (6), and *LEU4* (7). These genes have two in frame ATG codons near the 5' end of the open reading frame and at least two mRNA species are transcribed. The 5' end of the longer transcript maps upstream of the first AUG codon and that of the shorter one between the two AUG codons. The sequence between the two AUG codons encodes a targeting signal, thereby leading the proteins with and without the signal to distinct

locations. Such a mechanism was also found in the case of *Saccharomyces cerevisiae* CAT (8), whose longer transcript encodes the mitochondrial enzyme and shorter one, the peroxisomal enzyme. In the case of *MOD5* (9), on the other hand, all transcription initiation sites map upstream of the first AUG codon, suggesting that the shorter protein, initiating at the second AUG codon, is synthesized by a translational readthrough of the first AUG codon of the open reading frame. The mechanism involved in expression of CCA1 is more complicated (10). *CCA1* has three in frame AUG codons and multiple transcription initiation sites, upstream of the first AUG codon and between the first and second AUG codons. All three AUG codons are used as sites of initiation of translation. Therefore, CCA1 isozymes are targeted to three subcellular compartments, mitochondria, nucleus, and cytosol, by a combination of alternative transcription initiation and alternative translation initiation. Translational readthrough, or "leaky" ribosome scanning, is supposed to occur when the sequence near the first AUG codon shows diversity from a consensus sequence (Kozak configuration) for efficient initiation (11). Insufficient length of the 5' leader, or 5' untranslated region of the mRNA, is also given as a cause for leaky ribosome scanning (12). However, the precise mechanism responsible for leaky scanning has yet to be elucidated.

As described in this chapter, the results of primer extension analysis and RNase protection assay indicate that syntheses of mitochondrial and

peroxisomal CATs of *Candida tropicalis* were due to alternative translation initiation, which is the first case in yeast for an enzyme involved in intermediary metabolism (1). The effects of the sequences near the first AUG codon, between the first and second AUG codon, and the 5' leader were also examined on alternative translation initiation.

MATERIALS AND METHODS

1. Strains and vectors

Escherichia coli DH-5α was used as a host cell for cloning. *Saccharomyces cerevisiae* MT8-1 (13) was used as a host cell for the expression of the constructed plasmids.

2. Plasmid construction

Two primers with *Nco*I sites, 5'-AACCATGGTATCTCCTCTCTC-3' and 5'-TGCCATGGCTAAGTTGTAAGTCCG-3' were used in PCR with pWCAT1 (Part II, Chapter 1) as a template. By digesting the fragment with *Nco*I and then conducting self-ligation, a plasmid pWC1 containing the *CtCAT* 5' upstream region connected to the 3' downstream region with an *Nco*I junction was obtained. *Nco*I-*Nco*I fragments containing mutated *CtCAT* coding region were amplified by PCR with pWCAT1 as a template, using the primer 5'-ACTTAGCCATGGCATCAGTC-3' in combination with each of the following primers: 5'-AGATACCATGGCTTACACAAAGTTGTCG-

3' for pWCIL, 5'-GGAGATACCATGGCTAACTTTATGTTGTCG-3' for pWCDM, and 5'-ATTAACCATGGCCACCAAATCCA-3' for pWCSH. Each of the mutated coding region fragments was digested with *Nco*I and inserted into the *Nco*I site of pWC1 in the correct direction. Construction of the plasmid, pWICM1, was described in Part II, Chapter 2. pWCI1 and pWCI2 were constructed as follows. pWC2 was constructed by the same method as for pWC1 using the primers 5'-TGCCATGGTGGATTTGGTGGAA-3' and 5'-TGCCATGGCTAAGTTGTAAGTCCG-3'. The plasmid pWC2 containing the *CtCAT* 5'-upstream region and the region between the first and second ATG codons connected to the 3'-downstream region with an *Nco*I junction was obtained. An *Nco*I-*Nco*I fragment containing the *Candida tropicalis* isocitrate lyase gene was amplified with pMT34(-G7)-ICL(B1) (14) as a template using the primers 5'-CCTCCATTCCTCTTCTTGTC-3' and 5'-CTTTACCATGGCTTTTTTCTT-3'. This fragment was digested with *Nco*I and was inserted into the *Nco*I sites of pWC1 and pWC2. The plasmids obtained were named pWCI1 and pWCI2, respectively. All plasmids were transformed into *Saccharomyces cerevisiae* using the electroporation method as described in Part I, Chapter 1 (15).

3. Cultivation and preparation of cell-free extracts of *Candida tropicalis*

Candida tropicalis pK233 (ATCC 20336) was cultivated as in Part I, Chapter 1 (16). Cell-free extracts were prepared, as described Part I, Chapter 2.

4. Cultivation and preparation of cell-free extracts of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae bearing the constructed plasmid was cultivated as in Part I, Chapter 1. Cell-free extracts from *Saccharomyces cerevisiae* were prepared in the same manner as for *Candida tropicalis.*

5. Western blot analysis

Polyclonal antibodies against *Candida tropicalis* CAT (CtCAT) (17) were used to detect the enzyme. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out, as described in Part I, Chapter 1 (18). Protein was quantified by the modified Lowry method (19).

6. Isolation of RNA

Total RNA was extracted from *Candida tropicalis* cultured on acetate, glucose or *n*-alkane, or from *Saccharomyces cerevisiae* harboring various vectors cultured on oleic acid, as described by Kaiser *et al.* (20).

7. Primer extension

The oligonucleotide 5'-GGGTAATTGTGACTGGTATTTGAAC-3' was labeled with [γ-^{32}P] dATP and used as a primer for extension. The primer was annealed to 1-10 μg of total RNA and extended with Superscript II RNase H$^-$ reverse transcriptase (Bethesda Research Laboratories Life Technologies (BRL), Gaithersburg, MD, U.S.A.) at 42 ℃ for 30 min. The primer-extended products were separated on an 8 % polyacrylamide sequencing gel and detected by autoradiography. To provide a sizing ladder,

the same oligonucleotide was used for sequencing *CtCAT*.

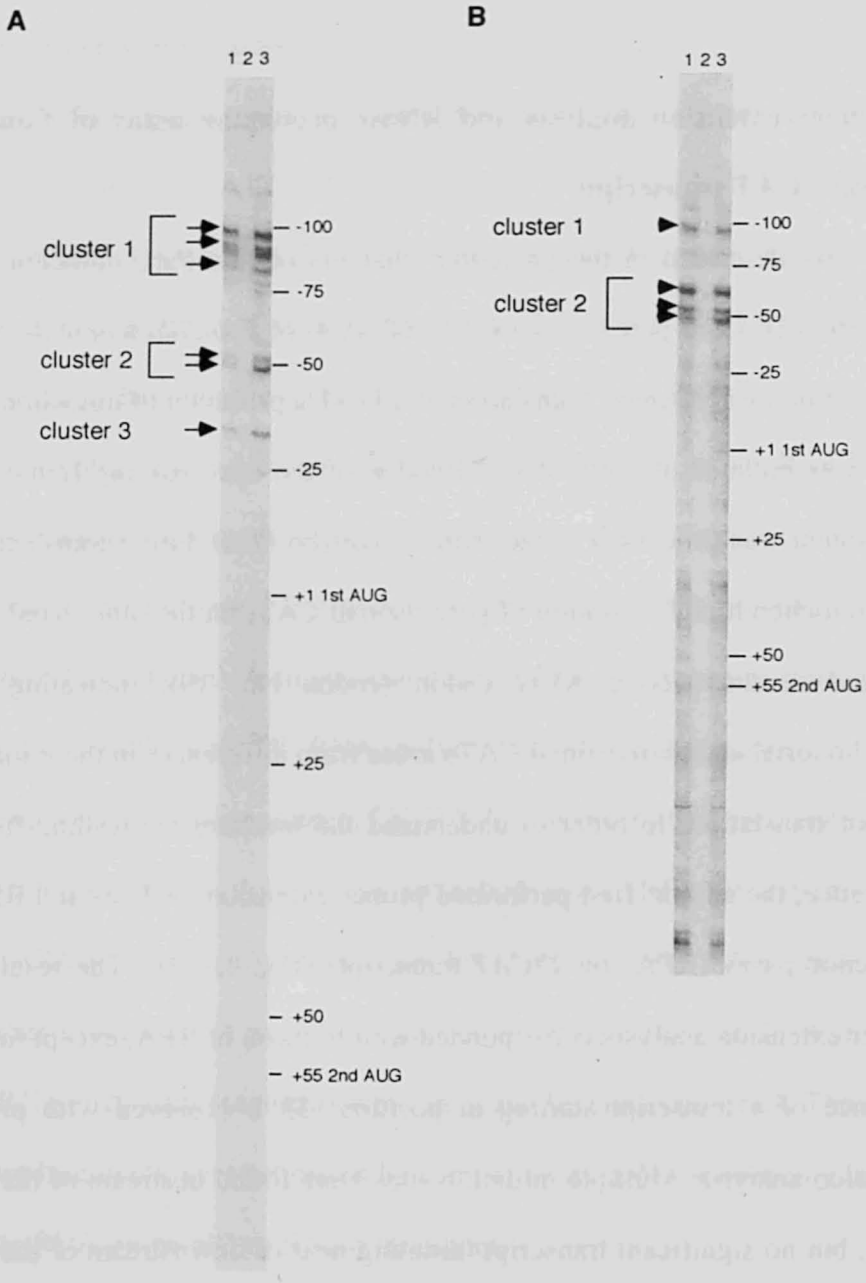
8. RNase protection assay

A 580 bp fragment was amplified using the oligonucleotides 5'-GGTCCATGGCTACATTTTTTCTTG-3' and 5'-CCGGCAGATCTTGGTC TAACAAAC-3' with pWCAT1 as a template. This fragment was digested by *Nco*I and *Bgl*II, and then was subcloned into the *Nco*I-*Bgl*II site of pMW1 (21), changing the *Kpn*I site to a *Bgl*II one by a *Bgl*II linker (Takara Shuzo, Kyoto, Japan), and the *Cla*I site to an *Nco*I site by an *Nco*I linker (Takara Shuzo). For synthesis of the RNA probe, the resulting plasmid was then linearized by *Sma*I. Restriction endonucleases were purchased from Toyobo (Osaka, Japan). A single-strand antisense RNA probe was transcribed using [α-^{32}P]UTP and T7 RNA polymerase of MAX1 script, *in vitro* transcription kit (Ambion, Austin, TX, U.S.A.), according to the recommended procedures by the vendor. RNase protection assay (RPA) was performed using RPAII, RNase protection assay kit (Ambion). The RNA probe was hybridized to 10 μg of total RNA and digested by RNase at 37 °C for 30 min. RNase resistant fragments were analyzed by electrophoresis. The sizes of these fragments were estimated by comparing the DNA sequencing ladder and the RNA probe before RNase treatment.

RESULTS

1. Primer extension analysis and RNase protection assay of *Candida tropicalis* CAT transcripts

As described in the preceding chapters of this Part, mitochondrial CAT (66 kDa) and peroxisomal CAT (68 kDa) of *Candida tropicalis* were products of a single gene. Translation of a 71 kDa precursor of mitochondrial CAT was initiated at the first AUG codon of the open reading frame, and the protein was processed to the mature size (66 kDa) during translocation into mitochondria. Translation of peroxisomal CAT, on the other hand, was initiated at the second AUG codon (codon No. 19), indicating that mitochondrial and peroxisomal CATs arose from differences in the initiation sites of translation. In order to understand the mechanisms leading to this difference, the author first performed primer extension analysis and RNase protection assay (RPA) on *CtCAT* transcripts (Fig. 1A, B). The results of primer extension analysis corresponded well to those of RPA, except for the presence of a transcript starting at position -33 b observed with primer extension analysis. Multiple initiation sites were found upstream of the first AUG, but no significant transcript initiating near or downstream of the first AUG codon could be detected. Transcription initiation sites upstream of the first AUG formed two clusters at positions around -90 b (cluster 1) and -50 b (cluster 2) (Fig. 2). The author categorized the transcript starting at position


A
1 2 3
cluster 1
cluster 2
cluster 3
-100
-75
-50
-25
+1 1st AUG
+25
+50
+55 2nd AUG
B
1 2 3
cluster 1
cluster 2
-100
-75
-50
-25
+1 1st AUG
+25
+50
+55 2nd AUG

C

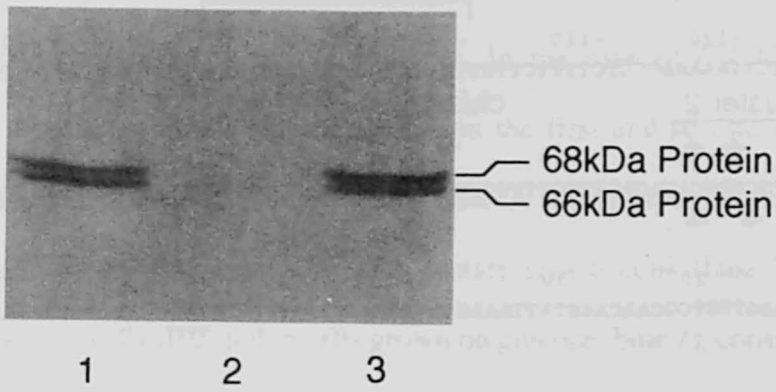


Fig. 1. Determination of the 5' ends of *CtCAT* transcripts (A, B) and Western blot analysis of cell-free extracts (C), from *Candida tropicalis* grown on various carbon sources. **A, B** Analysis of the 5' ends of *CtCAT* transcripts by primer extension (A) and RNase protection assay (B) are shown. Each analysis was performed on total RNA (10 μg) extracted from cells grown on acetate (lane 1), glucose (lane 2) or *n*-alkanes (lane 3). The numbers on the right were relative to A of the first AUG codon of the *CtCAT* transcripts. The arrows (A) and arrowheads (B) on the left indicate the positions of the major transcription initiation sites. **C** Cell-free extracts (30 μg) from acetate- (lane 1), glucose- (lane 2) or *n*-alkane-grown cells (lane 3) were applied to SDS-PAGE.

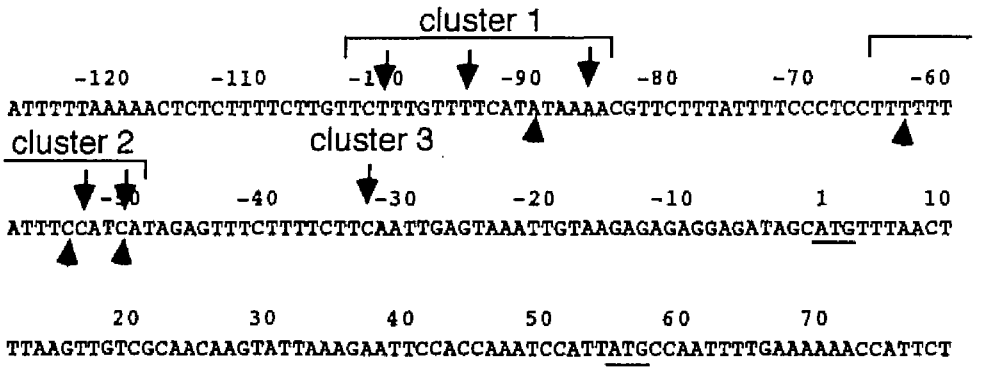


Fig. 2. Transcription initiation sites of *CtCAT*.

Multiple transcription initiation sites detected by primer extension and RNase protection assay are indicated by arrows and arrowheads, respectively. The first and second ATG codons of the open reading frame are underlined.

-33 b as cluster 3. In the case of *Saccharomyces cerevisiae* CAT (8), a shorter transcript initiating downstream of the first AUG, which encodes the peroxisomal isozyme, was specifically induced in oleate-grown cells, compared to acetate- or glycerol-grown cells. In the case of CtCAT, in contrast, no 5' mRNA ends was found between the first and second AUG (Fig. 1A, B) and it could not be detected any mRNA species specific to cells grown on *n*-alkanes when compared with acetate-grown cells (lane 1, 3). Transcripts were hardly detected in cells grown on glucose (lane 2), consistent with the low levels of CAT in these cells. The experiments described here were repeated 4 times with RNA isolated independently, all showing the same results. The distict results observed in transcription initiation sites of CAT mRNA between the two yeasts corresponded well to the differences of the CAT proteins. Contrary to *Saccharomyces cerevisiae* peroxisomal CAT, similar ratios of mitochondrial and peroxisomal CAT proteins of *Candida tropicalis* were detected in both acetate- and *n*-alkane-grown cells (Fig. 1C, lane 1, 3). In the case of *Candida tropicalis*, peroxisomal CAT was not specifically induced when cells were grown on *n*-alkane. The obtained results concerning the transcription and translation of CAT in *Candida tropicalis* strongly suggested that the peroxisomal enzyme, initiating at the second AUG codon, was generated by a translational readthrough of the first AUG codon of the open reading frame.

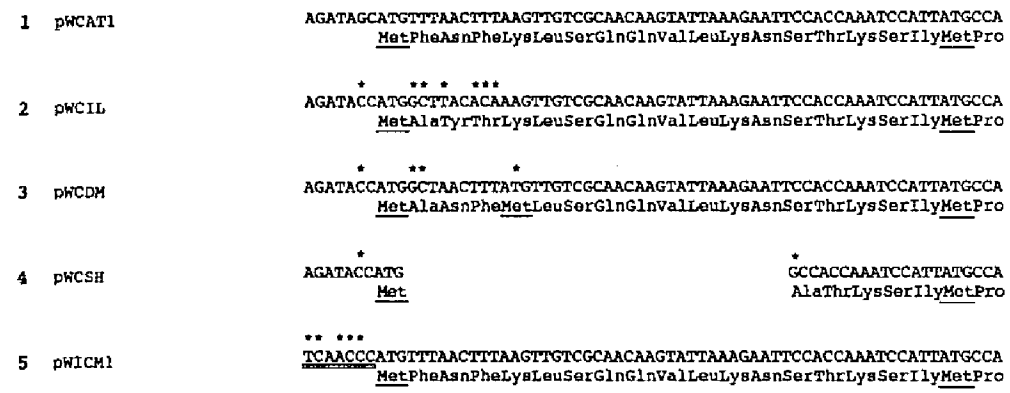


Fig. 3. Mutations introduced into the *CtCAT* gene.

The plasmids constructed were: pWCIL, whose sequence context near the first AUG of *CtCAT* imitated that of *Candida tropicalis* isocitrate lyase gene; pWCDM, mutated to introduce a new AUG between the first and second AUG; pWCSH, with a deletion of a major portion of the region between the first and second AUG codons; and pWICM1, whose 5' leader sequence was exchanged for *UPR-ICL*. The altered nucleotide sequences are indicated by asterisks. The in frame ATG codons of the open reading frame (Met) are underlined. *UPR-ICL* sequences were double underlined. Numbers for each plasmid correspond to the lane numbers in Fig. 4.

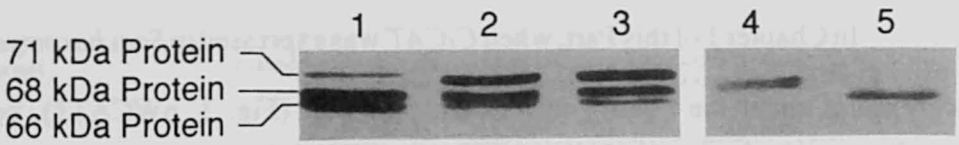
2. Alternative translation initiation of *CtCAT* transcripts in *Saccharomyces cerevisiae*

In Chapter 1 of this Part, when *CtCAT* was expressed in *Saccharomyces cerevisiae* under the control of its own promoter (Fig. 3, pWCAT1), both peroxisomal and mitochondrial CATs were synthesized (Fig. 4A, lane 1). Therefore, primer extension analysis of *CtCAT* mRNA was conducted with this yeast (Fig. 4B). Although the total number of mRNA species decreased, the author could detect major transcripts (transcripts 1, 2, and 3) corresponding well in length to the clusters 1, 2, and 3 (Fig. 1A) observed in the primer extension analysis of *Candida tropicalis* cells (Fig. 4B, lane 1). These transcripts could also be detected in four independent experiments. Other shorter transcripts seen in Fig. 4B were inconsistent in amount, suggesting that these were degradation products of the longer transcripts. The results indicate that alternative translation initiation of CtCAT also occurred in *Saccharomyces cerevisiae* and that the transcripts harbored sufficient information to bring about this phenomenon in both yeasts.

3. Effects of sequences near the first AUG codon on translation initiation

The mechanisms involved in alternative translation initiation have not yet been clarified in detail. According to the Kozak's scanning hypothesis (11), a ribosome recognizes the 5' end of a capped mRNA and migrates linearly to the 3' direction until it reaches the first AUG codon, where translation is initiated. Leaky ribosome scanning might be resulted from

A


1
2
3
4
5
71 kDa Protein
68 kDa Protein
66 kDa Protein

B

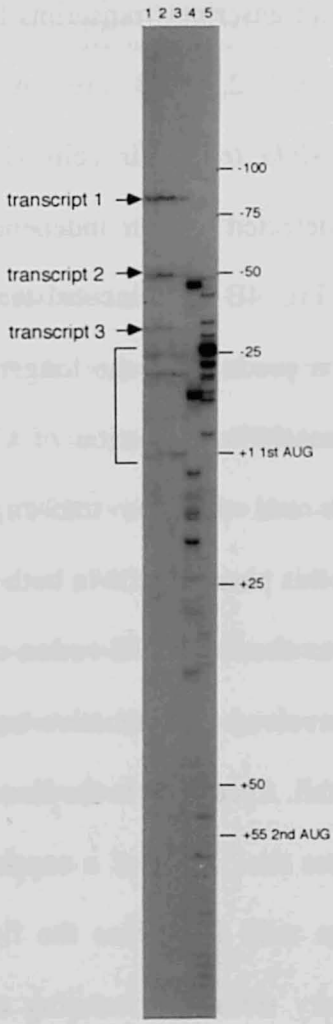

1 2 3 4 5
transcript 1
transcript 2
transcript 3
-100
-75
-50
-25
+1 1st AUG
+25
+50
+55 2nd AUG

Fig. 4. Western blot analysis of cell-free extracts (A), and determination of the 5' ends of transcripts (B), from cells harboring various mutated *CtCAT* genes.

A The respective proteins prepared from *Saccharomyces cerevisiae* harboring pWCAT1 (lane 1), pWCIL (lane 2), pWCDM (lane 3), pWCSH (lane 4), and pWICM1 (lane 5) were applied to SDS-PAGE. The amount of protein loaded on the gel was as follows: lane 1-3, 20 μg; lane 4 10μg, and lane 5, 1μg, respectively. **B** Primer extension analysis was performed on total RNA (lane 1-4, 10 μg each; lane 5, 1μg) extracted from *Saccharomyces cerevisiae* harboring pWCAT1 (lane 1), pWCIL (lane 2), pWCDM (lane 3), pWCSH (lane 4), and pWICM1 (lane 5). The numbers on the right are the same as in Fig. 1A, B. The arrows on the left indicate the positions of the major transcription initiation sites. The bracket indicates minor transcripts described in the text.

unfavorable Kozak configuration near the first AUG. From the comparison of many yeast mRNAs, 5'-(A/Y)A(A/U)AAUGUCU-3' has been proposed as a consensus sequence for efficient initiation (22). Using *Saccharomyces cerevisiae* as the host, the author investigated whether sequences near the first AUG codon influenced the efficiency of translation initiation. The author constructed pWCIL, leading to a transcript whose sequence context near the first AUG of *CtCAT* imitated that of *Candida tropicalis* isocitrate lyase gene, known to be strongly expressed in *Saccharomyces cerevisiae* (14) and pWCDM, mutated to introduce a new AUG between the first and second AUG in order to decrease the ratio of translation initiation at the second AUG (Fig. 3). The effects of these mutations on the translation products

were investigated by Western blot analysis (Fig. 4A). Owing to the introduction of the mutations, mitochondrial CAT, the product initiating from the first AUG codon, was not efficiently processed to the mature 66 kDa form and the majority remained as the 71 kDa precursor (lane 2, 3). The mutations probably decreased the efficiency of the N-terminal targeting signal. Therefore, the author compared the amount of the 68 kDa product with the sum of the 66 and 71 kDa products as the ratio of translation initiatiing from the second and first AUG codons. From the native *CtCAT* gene, equivalent amounts of the sum of the 66 and 71 kDa (first AUG), and 68 kDa (second AUG) products were observed (lane 1). In contrast, mutations in pWCIL and pWCDM led to a significant decrease in ratio of protein products initiating at the second AUG codon (68 kDa). The length and amount of transcripts from these mutant genes were identical with those from the native pWCAT1(Fig. 4B, lanes 1, 2, and 3), indicating that the ratio of protein products initiating at the first and second AUG codon could be changed independent of transcription. This confirms that the 66 (and 71 kDa) and 68 kDa proteins observed in *Saccharomyces cerevisiae* are also products due to alternative translation and that the 68 kDa protein is not a translation product of an mRNA initiating downstream of the first AUG codon.

4. Effects of the 5' leader and sequences between the first and second AUG codon on translation initiation

In the case of *MOD5*, the length of the leader has been shown to be

less than 11 b, and supposed to be too short for efficient translation initiation. When the native promoter was exchanged for *ADH1* promoter to produce a longer transcript, leaky scanning of the first AUG codon was abolished (12). It was suggested that the length of the leader sequence played an important role in alternative translation initiation of *MOD5* transcripts. When *CtCAT* was expressed using the *Candida tropicalis* isocitrate lyase promoter (*UPR-ICL*) (14) in *Saccharomyces cerevisiae*, the 68 kDa protein initiating at the second AUG codon could not be detected (Fig. 4A, lane 5) (Chapter 2 of this Part). Only the first AUG codon was recognized for initiation of translation. The 5' ends of transcripts from pWICM1 cells mapped mainly at -28 b (Fig. 4B, lane 5), which were further downstream of those of the shorter transcripts (transcript 3) detected from pWCAT1 cells (Fig. 4, lane 1). This indicates that in the case of *CtCAT*, not the length, but the sequence or secondary structure of the 5' leader sequence influenced translation efficiency at the first AUG codon.

To investigate whether other regions of the *CtCAT* mRNA influenced the initiation of translation at the first AUG codon, the author deleted a major portion corresponded to amino acid residues 2 to 13 of the region between the first and second AUG codon (pWCSH). The product shown in Fig. 4A, lane 4, corresponded to the expected molecular mass of the protein starting at the first AUG codon, slightly larger than the 68 kDa protein seen in lane 1, that was also confirmed by the results of electrophoresis for a long

time. Primer extension analysis showed that transcripts from pWCSH cells were initiated at the same sites as those from the native pWCAT1 cells, with differences in size corresponding to the length of deletion between the two AUG codons (Fig. 4B, lane 4). This indicates that the region between the first and second AUG codon is also necessary for alternative translation initiation. Regions further downstream of the second AUG codon had no effect on the alternative translation initiation. When the isocitrate lyase-encoding gene from *Candida tropicalis* as the other gene was fused directly downstream of the second ATG in frame (pWCI2) in comparison with the case without the region between the first and second ATG codons (pWCI1 in Fig. 5, lane 1), two products with molecular masses corresponding to the proteins from the first and second AUG codons were recognized (Fig. 5, lane 2).

DISCUSSION

The author determined the 5' ends of *CtCAT* transcripts using primer extension analysis and RPA. All 5' ends of transcripts appeared upstream of the first AUG codon, regardless of growth conditions (Fig. 1A, B). This strongly suggested that peroxisomal and mitochondrial CAT of *Candida tropicalis* were synthesized from *CtCAT* by alternative translation initiation (Fig. 6). These results are distinct from the mitochondrial and peroxisomal

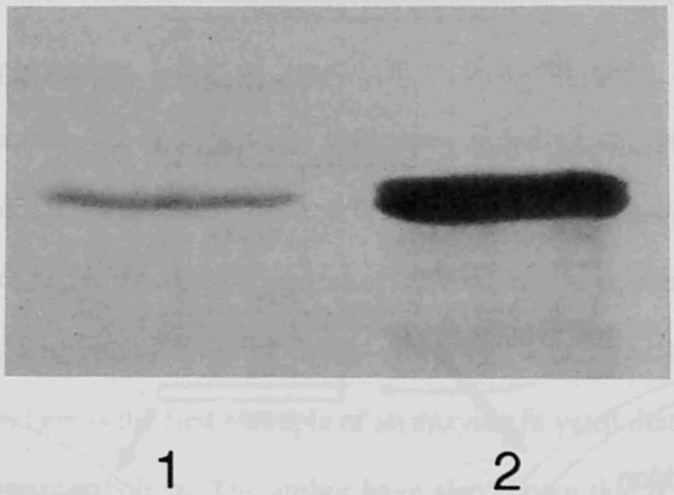


Fig. 5. Western blot analysis of cell-free extracts prepared from *Saccharomyces cerevisiae* harboring pWCI1 (lane 1) and pWCI2 (lane 2).

Twnty μg proteins were applied to SDS-PAGE. Construction of pWCI1 and pWCI2 was described in MATERIALS AND METHODS.

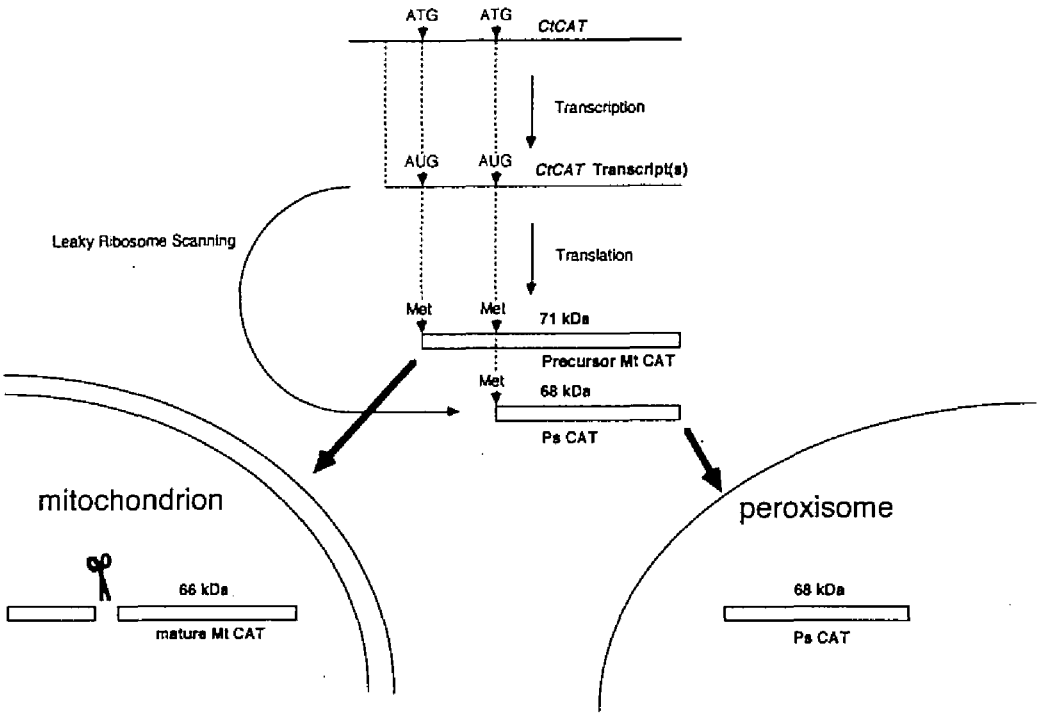


Fig. 6. Schematic figure of the generation of peroxisomal and mitochondrial CATs in *Candida tropicalis*. Mt, mitochondrial; Ps, peroxisomal.

CATs of *Saccharomyces cerevisiae*, where dual organelle localization is attributable to alternative transcription initiation (8).

There have been only two reports that alternative translation initiation has been suggested to occur in yeast. These two examples, *MOD5* and *CCA1* of *Saccharomyces cerevisiae*, are genes encoding enzymes required for tRNA synthesis, which are localized in mitochondria, nucleus and cytosol (9, 10). In both cases, the short 5' leaders of their mRNA have been suggested to be mainly responsible for the leaky ribosome scanning. The case of CtCAT shown here is the first example of an enzyme in yeast that is involved in intermediary metabolism. The author have also shown that it was not the length of the 5' leader, but the sequence context of the region from the transcription initiation site to the second AUG codon that contributed to the alternative translation initiation.

Alternative translation initiation is not restricted to yeast and has been reported in higher eukaryotes, such as in the cases of rat liver mitochondrial and cytosolic fumarase (23) and feline mitochondrial and peroxisomal alanine:glyoxylate aminotransferase (24). In the case of fumarase, it has been suggested that the secondary structure of the 5' leader may hinder the initiation from the first AUG codon, resulting in the second AUG codon more accessible than the first. However, experimental evidence at present is not sufficient to give light to the precise mechanisms involved. The author constructed various mutant *CtCAT* genes to define factors that

influence the efficiency of alternative translation initiation (Fig. 3). The results indicated that the 5' leader and the region between the first and second AUG codons were prerequisite for the alternative translation initiation. Mutations in the sequence near the first AUG codon decreased the ratio of leaky ribosome scanning, but not completely. There have been a previous report indicating that the sequence near the AUG codon in yeast has a less significant effect on the efficiency of translation initiation than in higher eukaryotes (25). The results suggest that the 5' leader and the region between the first and second AUG codons may interact with one another to form secondary structures leading to a translational readthrough of the first AUG codon. The diversity of the sequence near the first AUG codon from the consensus sequence for efficient translation may also enhance this tendency. The author analyzed the secondary structure of CtCAT, according to Zuker and Stiegler (26), but could not clarify these suggestions.

From the results of primer extension analysis and RPA of CtCAT, a large number of transcripts could be detected. At present, the author can not distinguish the function of each transcript. However, as the result of alternative translation initiation, the steady-state levels of mitochondrial and peroxisomal CATs of *Candida tropicalis* were approximately equal. The question remains whether each protein product is derived from a specific transcript or whether each transcript is subjected to equal ratios of alternative translation initiation. It will be further attractive to define the protein product(s) encoded by each

transcript in order to determine the presence of an mRNA specific for the peroxisomal isozyme.

SUMMARY

Carnitine acetyltransferase (CAT; EC 2.3.1.7) is localized in two subcellular organelles, mitochondria and peroxisomes, in an *n*-alkane-assimilating yeast, *Candida tropicalis*. The isozymes are synthesized from the first and second ATG codon of the open reading frame of one gene, *CtCAT*. Primer extension analysis and RNase protection assay revealed that multiple transcription initiation sites were found upstream of the first ATG codon. No 5' ends could be detected between the first and second AUG codons. These results strongly suggested that the peroxisomal CAT of *Candida tropicalis*, initiating at the second AUG codon of the transcripts, was synthesized by a translational readthrough of the first AUG codon of the open reading frame. When *CtCAT* was introduced into the yeast, *Saccharomyces cerevisiae*, 5' ends of transcripts and the protein products were similar to those observed in *Candida tropicalis* CAT. This suggested that the transcripts harbored sufficient information to bring about alternative translation initiation in both yeasts. Using *Saccharomyces cerevisiae* as the host cell, introduction of mutations into the sequence near the first AUG codon or a deletion in the region between the first and second AUG codons

resulted in an increased ratio of translation from the first AUG codon, although initiation sites of transcription did not change. Moreover, replacing the 5' leader sequence to that of *Candida tropicalis* isocitrate lyase promoter (*UPR-ICL*), eliminated the product initiating at the second AUG codon. The transcript from these cells was shorter than those detected from the native *CtCAT*-harboring cells, suggesting that the alternative translation initiation of *Candida tropicalis* CAT was mainly dependent on the structure and sequence context of the region from the 5' end to the second AUG codon, and not the insufficient length of the 5' leader.

REFERENCES

1. Danpure C.J. (1995) *Trends Cell Biol.* **5**, 230.
2. Carlson, M. & Botstein D. (1982) *Cell* **28**, 145.
3. Natsoulis, G., Hilger, F. & Fink, G.R. (1986) *Cell* **46**, 235.
4. Wu, M. & Tzagoloff, A. (1987) *J. Biol. Chem.* **262**, 12275.
5. Chatton, B., Walter, P., Ebel, J.-P., Lacroute, F. & Fasiolo, F. (1988) *J. Biol. Chem.* **263**, 52.
6. Ellis, S.R., Hopper, A.K. & Martin N.C. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5172.
7. Beltzer, J.P., Morris, S.R. & Kohlham, G.B. (1988) *J. Biol. Chem.* **263**, 368.

8. Elgersma, Y., Van Roermund, C.W.T., Wamders, R.J.A. & Tabak, H.F. (1995) *EMBO J.* **14**, 3472.

9. Najarian, D., Dihanich, M.E., Martin, N.C. & Hopper, A.K. (1987) *Mol. Cell. Biol.* 7, 185.

10. Wolfe, C.L., Lou, Y.-C., Hopper, A.K. & Martin, N.C. (1994) *J. Biol. Chem.* **269**, 13361.

11. Kozak, M. (1991) *J. Biol. Chem.* **266**, 19867.

12. Slusher, L.B., Gillman, E.D., Martin, N.C. & Hopper, A.K. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9789.

13. Tajima, M., Nogi, Y. & Fukasawa, T. (1985) *Yeast* **1**, 67.

14. Umemura, K., Atomi, H., Kanai, T., Teranishi, Y. Ueda, M. & Tanaka, A. (1995) *Appl. Microbiol. Biotechnol.* **43**, 489.

15. Brecker, D.M. & Guarente, L. (1991) *Methods Enzymol.* **194**, 182.

16. Ueda, M., Okada, H., Tanaka, A., Osumi, M. & Fukui, S. (1983) *Arch. Microbiol.* **136**, 169.

17. Ueda, M., Tanaka, A. & Fukui, S. (1984) *Eur. J. Biochem.* **138**, 445.

18. Ueda, M., Mozaffar, S., Atomi, H., Osumi, M. & Tanaka, A. (1989) *J. Ferment. Bioeng.* **68**, 411.

19. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.

20. Kaiser, C., Michaelis, S. & Mitchell, A. (1994) *Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press.*

21. Kanai, T., Atomi, A., Umemura, K., Ueno, H., Teranishi, Y., Ueda, M. & Tanaka, A. (1996) *Appl. Microbiol. Biotechnol.* **44**, 759.

22. Cigan, A.M. & Donahue, T.F. (1987) *Gene* **59**, 1.

23. Suzuki, T., Yoshida, T. & Tuboi, S. (1992) *Eur. J. Biochem.* **207**, 767.

24. Lumb, M.J., Purdur, P.E. & Danpure, C.J. (1994) *Eur. J. Biochem.* **221**, 53.

25. Kozak, M. (1991) *J. Cell Biol.* **115**, 887.

26. Zuker, N. & Stiegler, P. (1981) *Nucleic Acids Res.* **9**, 133.

GENERAL CONCLUSION

The present study has been carried out to obtain some basal biochemical information on the isozymes, NADP-IDHs and CATs, localized in both peroxisomes and mitochondria of *n*-alkane-utilizable yeast *Candida tropicalis*.

The author could show the presence of mitochondrial NADP-IDH in *Candida tropicalis* and its nucleotide sequence. From the results of Western blot analysis and immunochemical titration experiments, mitochondrial and peroxisomal NADP-IDHs were immunochemically distinguishable from each other, suggesting that these isozymes are encoded by distinct genes.

The gene for peroxisomal NADP-IDH was isolated using the cDNA fragment specific for this enzyme as a probe and sequenced. This was the first case of sequencing of a peroxisomal NADP-IDH gene. Although the C-terminal sequence of this enzyme did not meet a proposed peroxisomal targeting signal, it had sequences near the N-terminal region with a high similarity with both the putative N-terminal peroxisomal tergeting sequence of 3-ketoacyl-CoA thiolase of *Saccharomyces cerevisiae* and an internal region of acyl-CoA oxidase of *Candida tropicalis*.

The author also succeeded in the isolation of one gene encoding *Candida tropicalis* CAT. From the result of the expression in *Saccharomyces cerevisiae*, the CAT isozymes were revealed to be encoded by this gene.

Furthermore, the distinct translational initiation sites of the peroxisomal and mitochondrial CATs have been proposed.

Candida tropicalis peroxisomal and mitochondrial CAT-encoding genes were expressed individually in *Saccharomyces cerevisiae* using the isocitrate lyase promoter from *Candida tropicalis*. The 71 kDa precursor of mitochondrial CAT, initiating at the first Met, was converted to the mature size (66 kDa) after translocation into mitochondria. On the other hand, 68 kDa peroxisomal CAT, initiating at the second Met, was not processed and was translocated into peroxisomes and cytosol, but not into mitochondria. These results demonstrated that mitochondrial and peroxisomal CATs arose from differences in the initiation sites of translation.

Next, the 5' ends of transcripts of *Candida tropicalis* CAT was determined. Primer extension analysis and RNase protection assay indicated that alternative translation initiation was responsible for the sorting of CATs to peroxisomes and mitochondria. It was showed that not the insufficient length of the 5' leader but the sequence context of the regions surrounding the first AUG codon have a major role in alternative translation initiation of *Candida tropicalis* CATs.

PUBLICATION LIST

Part I

Chapter 1

Immunochemically distinct NADP-linked isocitrate dehydrogenase isozymes in mitochondria and peroxisomes of *Candida tropicalis*

Imajo, T., Kawachi, H., Atomi, H., Sanuki, S., Yamamoto, S., Ueda, M. & Tanaka, A.

Arch. Microbiol., in press.

Chapter 2

Gene analysis of a novel NADP-linked isocitrate dehydrogenase localized in peroxisomes of the *n*-alkane-assimilating yeast *Candida tropicalis*

Kawachi, H., Shimizu, K., Atomi, H., Sanuki, S., Ueda, M. & Tanaka, A.

Eur. J. Biochem., in contribution.

Part II

Chapter 1

Peroxisomal and mitochondrial carnitine acetyltransferases of the *n*-alkane-assimilating yeast *Candida tropicalis*: analysis of gene structure and translation products

Kawachi, H., Atomi, H., Ueda, M. & Tanaka, A.

Eur. J. Biochem. (1996) **238**, 845-852

Chapter 2

Individual expression of *Candida tropicalis* peroxisomal and mitochondrial carnitine acetyltransferase-encoding genes and subcellular localization of the products in *Saccharomyces cerevisiae*

Kawachi, H., Atomi, H., Ueda, M., Hashimoto, N., Koboyashi, K., Yoshida, T., Kamasawa, N., Osumi, M. & Tanaka, A.

J. Biochem. (1996) **120**, 731-735

Chapter 3

Alternative translation initiation from the transcripts of a single gene encoding mitochondrial and peroxisomal carnitine acetyltransferases of the *n*-alkane-assimilating yeast, *Candida tropicalis*

Kawachi, H., Atomi, H., Ueda, M. & Tanaka, A.

Eur. J. Biochem., in contribution.

Other Publications

Characterization of the intron-containig citrate synthase gene from the alkanotrophic yeast *Candida tropicalis*: cloning and expression in *Saccharomyces cerevisiae*

Ueda, M., Sanuki, S., Kawachi, H., Shimizu, K., Atomi, H. & Tanaka, A.

Arch. Microbiol., in press.